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RELATION BETWEEN PARASITIZATION OF TWIG-INFESTING LARVAE OF THE ORIENTAL FRUIT MOTH AND SUBSEQUENT INFESTATION OF RIPE PEACHES[1] [2]

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INTRODUCTION

Information on the effect of parasites in reducing the infestation of the peach crop by the oriental fruit moth (*Grapholitha molesta* (Busck)) is important for determining the advisability of continued widespread liberations of these parasites. In this study an attempt has been made to show whether a relation can be detected between parasitization[3] in twigs at the peak of second-brood infestation and the total moth infestation of ripe peaches during the harvesting period for Elberta and other varieties ripening at about the same time. In the section of the country in which these observations were made, second-brood infestation of twigs usually occurs from the latter part of June until about the middle of July, and ripe-fruit infestation of Elberta peaches from about the first of August to the first week in September.

REVIEW OF LITERATURE

One rather intensive study of the relation between parasitization and fruit infestation was made by Daniel[4] in Niagara County, N. Y., from 1928 to 1931, inclusive. Daniel noted that fully one-third of the fruit infestation was not visible externally, and the data in his paper indicate that such injury ranged from less than 40 percent to more than 300 percent of the visible injury. He commented that this condition makes valueless any counts that do not take it into consideration and concluded that a large portion of the observed reduction in fruit infestation was caused by parasites. This work in Niagara County was continued during 1932 and 1933, and in other reports by Daniel and coworkers[5] [6] the conclusions were essentially the same as previously noted.

In 1940 Yetter and Allen[7] reported a study of larval parasitization and ripe-fruit infestation in eight orchards in Burlington County,

[1] Received for publication November 24, 1942.

[2] Many individuals had a part in accumulating the data used in this paper. Information for the New Jersey orchards was collected by M. H. Brunson and W. P. Yetter; the rearing of twig collections for the record of parasitization was done by G. J. Haeussler; the field work in Virginia, West Virginia, and Maryland and the assembling of the data were done with the assistance of E. L. Plasket and D. W. Clancy.

[3] The term "parasitization" as used in this paper signifies parasitization of twig-infesting larvae of the second brood of the oriental fruit moth. A number of species of parasites were involved, but the most important species was usually *Macrocentrus ancylivorus* Roh.

[4] DANIEL, D. M. MACROCENTRUS ANCYLIVORUS ROHWER, A POLYEMBRYONIC BRACONID PARASITE OF THE ORIENTAL FRUIT MOTH. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bul. 187, 101 pp., illus. 1932.

[5] DANIEL, D. M., COX, J., and CRAWFORD, A. BIOLOGICAL CONTROL OF THE ORIENTAL FRUIT MOTH. N. Y. (Geneva) Agr. Expt. Sta. Bul. 635, 27 pp., illus. 1933.

[6] DANIEL, D. M. UTILIZING PARASITES IN CONTROLLING THE ORIENTAL FRUIT MOTH. Ent. Soc. Amer. Ann. 29: 640–644. 1936.

[7] YETTER, W. P., JR., and ALLEN, H. W. EFFECT OF LARVAL PARASITIZATION OF THE ORIENTAL FRUIT MOTH ON THE INFESTATION. Jour. Econ. Ent. 33: 349–353. 1940.

N. J. In the group of orchards having the higher rates of parasitization the average infestation was much less than in the group having the lower rates. In the same year Brunson [8] published the results of mass liberation experiments in Mercer County, N. J., in which he made intensive observations on the parasitization and moth population in five orchards. These experiments also indicate that high parasitization is usually associated with low fruit infestation.

A few other workers have made observations on the relation of parasitization to fruit infestation, but most of these observations have been based on total emergence of relatively small numbers of insects, or on fruit counts in which the highly variable portion of the total injury invisible externally was not accurately determined by cutting fruit samples.

EXPERIMENTAL DATA AND ITS SOURCE

The relation between parasites and fruit infestation is thus seen to be essentially one of the interrelation of populations. To express fully this relationship in any given orchard it would be necessary to obtain information on the weekly fluctuations in parasite and moth populations per unit area. It would also be necessary to measure the effect of various other factors, such as unfavorable weather, cocoon and egg parasites, predators, unfavorable twig growth, lack or scarcity of fruits, control measures used by the grower, changes produced by migration to or from the orchard—considering them all in relation to the moth population at the beginning of the season. It is obviously impossible in the conduct of any small project to obtain such complete information for a large number of orchards. However, if the sum of all the unmeasured factors affecting the population of the fruit moth tends to be so small as to be overshadowed by the factor of parasitization, or, if not small, shows some degree of uniformity for orchards over a certain area or during a certain season, the true effect of such parasitization will become apparent without adjustment for these other factors. The study reported here has been based on this assumption.

The data used in this study are the records from the same orchards and for the same seasons as were used by Yetter and Allen [9] and Brunson [10], and, in addition, from a larger series of orchards, including 11 peach-growing districts, extending from Lovingston, Va., to Princeton, N. J., for the 3 years 1937 to 1939, inclusive. In the New Jersey orchards parasitization was determined from large numbers of infested twigs collected over 4-day periods, as described by Yetter and Allen. In the other orchards the parasitization was determined from two collections of twigs in each orchard at or near the peak of infestation for the second brood of fruit-moth larvae. The fruit records were based on samples of 300 to 400 peaches taken from each orchard at harvesttime, all of which were cut open. The data differ in one important respect from those obtained by Daniel and his associates—that is, they provide a basis for the comparison of different orchards in any year—whereas Daniel's comparisons are chiefly of the differences during several successive years for one district.

[8] BRUNSON, M. H. MASS LIBERATIONS OF PARASITES OF THE ORIENTAL FRUIT MOTH FOR IMMEDIATE REDUCTION OF INFESTATION. Jour. Econ. Ent. 33:346-349. 1940.
[9] See footnote 7.
[10] See footnote 8.

These observations, which include 87 summations of orchard parasitization and fruit infestation obtained from 51 orchards, are presented in table 1, and furnish the data upon which the subsequent comparisons are made.

TABLE 1.—*Surveys of parasitization of second-brood larvae of the oriental fruit moth in infested twigs and the injury to ripe peaches, 1937–39*

1937 SURVEY

District	Orchard No.	Fruit moth larvae reared from samples	Larvae parasitized	Bearing trees per acre	Fruits harvested per tree	Fruits injured			
						Relative injury		Fruits per tree	
						New	Total	New	Total
		Number	*Percent*	*Number*	*Number*	*Percent*	*Percent*	*Number*	*Number*
Virginia:	1	187	56.7	106	728	5.3	8.3	39	60
Lovingston	2	87	4.6	139	779	3.5	6.5	27	51
	3	83	8.4	128	587	4.3	6.8	25	40
	7	45	82.2	49	678	2.5	3.3	17	22
Staunton	8	47	2.1	91	365	12.3	15.3	45	56
	9	55	5.5	75	378	11.0	14.0	42	54
Harrisonburg	11	337	5.9	101	54	37.8	46.3	20	25
Timberville	15	229	9.0	59	841	7.0	9.0	59	76
	16	76	1.3	98	742	3.3	3.8	25	28
	17	70	0	118	596	4.5	5.5	27	33
Clear Brook	19	29	0	92	655	2.5	4.0	16	26
	20	111	2.7	37	326	22.5	31.3	73	102
Maryland:									
Smithsburg-Ringgold	30	274	55.8	161	901	1.7	2.1	16	19
	31	49	69.4	134	126	2.5	6.0	3	8
	33	47	63.8	69	1,411	2.3	3.3	32	47
	35	595	46.9	119	612	6.0	8.6	37	53
New Jersey:									
Moorestown	40	1,767	71.0	102	1,904	9.9	12.2	188	232

1938 SURVEY

Virginia:									
Crozet-Afton	4	67	95.6	91	583	1.3	4.0	8	24
	6	56	96.4	104	631	1.3	2.0	8	12
Staunton	8	93	15.1	39	200	12.0	19.5	24	40
	9	153	5.2	83	92	28.6	42.0	26	40
Harrisonburg	11	85	12.9	101	400	37.6	47.3	150	189
	12	71	25.4	53	619	22.0	26.6	136	165
Timberville	14	135	48.9	70	118	22.0	34.6	26	41
	15	111	27.0	101	683	27.2	32.2	187	222
	16	92	32.6	133	355	12.8	14.5	47	53
Clear Brook	20	36	13.9	30	137	10.0	12.2	14	17
	21	17	29.4	105	313	7.7	9.7	27	34
West Virginia:									
Martinsburg	23	131	58.8	95	418	9.8	15.0	42	65
	24	128	46.1	38	533	17.0	26.0	87	136
	25	134	88.8	107	957	7.5	7.5	71	71
Maryland:									
Hancock	27	75	42.7	109	274	6.2	14.2	17	39
	28	63	93.7	78	195	1.3	1.6	2	3
	29	60	90.0	132	293	2.7	3.7	8	11
Smithsburg-Ringgold	30	32	65.6	152	407	1.7	1.7	8	8
	32	70	98.6	120	1,004	.6	.6	6	6
	34	3	0	41	125	6.0	7.2	8	10
New Jersey:									
Moorestown	36	283	57.2	58	349	21.2	27.2	80	100
	37	1,181	60.1	75	367	27.5	31.0	104	117
	38	210	63.2	85	225	2.5	3.7	5	8
	41	467	86.1	76	394	3.5	4.7	15	20
	42	132	88.9	103	411	2.3	3.0	10	13
	43	952	72.7	78	436	11.8	21.0	51	93
	44	436	77.4	112	227	15.0	17.5	34	40
	45	679	84.5	90	139	9.2	16.2	13	23
Mercer County	47	574	84.8	102	75	19.0	26.2	14	20
	48	782	83.1	90	553	4.5	5.2	25	30
	49	3,268	59.6	86	387	15.0	17.5	58	69
	50	108	85.5	109	163	10.2	10.7	17	18
	51	251	79.0	107	660	21.7	23.2	147	158

TABLE 1.—*Surveys of parasitization of second-brood larvae of the oriental fruit moth in infested twigs and the injury to ripe peaches, 1937–39*—Continued

1939 SURVEY

District	Orchard No.	Fruit moth larvae reared from samples	Larvae parasitized	Bearing trees per acre	Fruits harvested per tree	Fruits injured			
						Relative injury		Fruits per tree	
						New	Total	New	Total
Virginia:		*Number*	*Percent*	*Number*	*Number*	*Percent*	*Percent*	*Number*	*Number*
Lovingston	1	220	12.7	100	100	44.7	50.7	45	51
	3	215	8.8	134	753	18.1	18.4	136	138
Crozet-Afton	4	166	16.3	94	888	3.8	3.8	34	34
	5	181	11.6	106	583	4.1	4.8	24	28
Staunton	6	124	23.4	105	558	1.0	1.3	6	7
	10	221	48.0	78	460	6.0	7.7	27	35
Harrisonburg	11	241	2.9	101	311	32.2	39.2	100	122
	12	147	8.8	53	280	22.2	29.9	62	84
Timberville	13	257	16.0	106	294	14.7	17.4	43	51
	16	215	3.7	132	218	7.5	9.1	16	20
	18	216	3.7	102	490	18.9	22.7	92	111
Clear Brook	20	197	43.1	41	321	5.4	6.4	17	21
	21	176	25.6	32	582	12.3	14.2	71	82
	22	157	68.8	100	448	1.3	2.3	6	11
West Virginia:									
Martinsburg	23	168	81.0	97	1,102	2.3	2.3	25	25
	25	178	81.5	103	1,173	1.3	1.7	15	20
	26	202	57.9	60	1,162	1.3	2.7	15	31
Maryland:									
Hancock	27	165	84.8	111	237	1.1	1.1	2	3
	28	24	72.4	76	591	0	0	0	0
	29	1	100.0	139	309	.7	.7	2	2
Smithsburg-Ringgold	30	30	46.7	171	888	2.7	2.7	24	24
	32	31	80.6	126	626	.3	.8	2	5
	34	18	61.1	44	166	9.3	9.3	15	16
New Jersey:									
Moorestown	36	69	47.8	58	556	.....	21.3	.....	118
	37	258	46.9	61	497	.....	34.5	.....	171
	38	409	85.8	81	669	.....	3.9	.....	26
	39	482	90.0	138	419	.....	12.7	.....	53
	41	454	82.2	72	543	.....	5.8	.....	31
	42	250	58.4	105	124	.....	15.4	.....	19
	43	293	71.0	79	882	.....	7.9	.....	65
	45	152	85.5	87	646	.....	4.6	.....	30
	46	578	32.9	109	720	.....	35.0	.....	252
Mercer County	47	1,361	84.6	91	224	.....	21.1	.....	47
	48	313	92.3	105	898	.....	6.5	.....	59
	49	2,703	90.0	78	668	.....	42.5	.....	284
	50	176	94.3	109	467	.....	14.5	.....	68
	51	44	59.1	105	531	.....	31.6	.....	168

DISCUSSION OF DATA

For the determination of parasitization a total of 26,115 insects were reared from collections of infested twigs. In a few orchards, such as No. 34 in 1938 and No. 29 in 1939, the numbers reared are too small to permit accurate determination of the percentage parasitized for the orchard. In some orchards, as in Nos. 11 and 40 in 1937, the fruit load per tree departs widely from the normal. Since the tendency is toward large numbers of injured fruits and low percentages of injury with full crops and toward the converse with partial crops, such wide variations in crop size affect the measure of fruit infestation whether based on the numbers of injured fruits per tree or on the percentage of fruit injured. Despite the deficiencies and irregularities noted for some of the orchards, it is more desirable to include them in class groupings for the purpose of obtaining averages than to attempt arbitrarily to exclude some and to include others.

The values for fruit injury in table 1 include new injury and total injury. New injury is produced by the progeny of the moths that

survive second-brood parasitization, and is therefore a better value for comparison with such parasitization than total injury, which includes older injury produced by larvae unaffected by second-brood parasitization. However, since determinations of new injury were not available for all orchards, total injury has been taken as the measure of fruit infestation in subsequent tables.

In table 2 the data for all the orchards from which comparable records were obtained are averaged for five classes, each covering a range of 20 percent of parasitization. In this arrangement there is no well-defined relation between the percentage of parasitization and the average fruit infestation. However, the class having 80- to 100-percent parasitization has the fewest injured fruits per tree and the lower percentage of injury. Only 3 observations in this class (No. 25 in 1938 and Nos. 49 and 50 in 1939) show more injured fruits per tree than the average of the lowest class (59.4), and only 1 (No. 49 in 1939) shows evidence of having, both in the number of injured fruits per tree and in percentage of injury, a greater degree of injury than the average of the classes of lower parasitization. On the other hand, in the 4 classes of less than 80-percent parasitization many observations show a fruit infestation lower than the average for the group having 80- to 100-percent parasitization. In the class of 60- to 79.9-percent parasitization 6 of the 13 observations are of this type, and in the successively lower classes the numbers are 6 out of 17, 2 out of 7, and 9 out of 24.

TABLE 2.—*Observations for second-brood parasitization in twigs and the total injury to peaches at harvesttime, averaged for 5 classes of parasitization*

Second-brood larvae parasitized, class range, in percent	Observations	Average injured fruits	Injured fruits per tree	Second-brood larvae parasitized, class range, in percent	Observations	Average injured fruits	Injured fruits per tree
	Number	*Percent*	*Number*		*Number*	*Percent*	*Number*
80–100.0	26	8.0	35.6	20–39.9	7	20.0	116.4
60–79.9	13	10.7	61.8	0–19.9	24	19.4	59.4
40–59.9	17	16.2	68.8				

The probable explanation of this condition is that in orchards showing exceptionally high parasitization it is difficult for the fruit moth to increase, even in the absence of other controlling factors, whereas in orchards showing lower parasitization these other factors may frequently be of greater importance in determining the degree of fruit injury.

In 32 of the 51 orchards surveyed there are observations for 2 or 3 seasons. When the parasitization and the number of infested fruits are compared for each of the 32 orchards, the lower infestations are found to be associated with higher parasitization in the expected relation in only 12 orchards. When expressed in percentages, lower infestation is similarly associated with higher parasitization in only 14 orchards. It is therefore evident that in any orchard the occurrence of a parasitization higher or lower than that of the previous year is not necessarily followed by an equivalent change in the opposite direction in the amount of fruit infestation. Either the observed parasitization was unimportant in the control of the fruit moth, or its true importance was obscured by a high seasonal variability in the sum of the other factors determining moth abundance at harvesttime.

The comparison of data for individual orchards for successive years indicates that the relation between parasitization and fruit infestation may be obscured by important seasonal differences in the level of fruit moth populations, brought about by factors other than parasitism. In table 1 it is seen that in both 1938 and 1939 the levels of parasitization and of fruit infestation in New Jersey were quite different from those in the other districts surveyed. For these reasons a better basis for comparing parasitization with fruit infestation is obtained by restricting the comparisons within series to observations during the same year and for an area over which the sum of control agencies other than parasitism does not vary greatly.

TABLE 3.—*Injury to peaches by the oriental fruit moth in relation to the degree of parasitization of second-brood larvae as determined by orchards in Virginia, West Virginia, Maryland, and New Jersey in 1937, 1938, and 1939; orchards in each group arranged in order of decreasing parasitization*

1937 survey				1938 survey				1939 survey			
Orchard No.	Second-brood parasitization	Total injured fruits	Injured fruits per tree	Orchard No.	Second-brood parasitization	Total injured fruits	Injured fruits per tree	Orchard No.	Second-brood parasitization	Total injured fruits	Injured fruits per tree
Virginia and Maryland:	*Pct.*	*Pct.*	*No.*	Virginia, West Virginia, and Maryland:	*Pct.*	*Pct.*	*No.*	Virginia, West Virginia, and Maryland:	*Pct.*	*Pct.*	*No.*
7	82.2	3.3	22	32	98.6	0.6	6	27	84.8	1.1	3
31	69.4	6.0	8	6	96.4	2.0	12	25	81.5	1.7	20
33	63.8	3.3	47	4	95.6	4.0	24	23	81.0	2.3	25
1	56.7	8.3	60	28	93.7	1.6	3	32	80.6	.8	5
30	55.8	2.1	19	29	90.0	3.7	11	28	72.4	0	0
35	46.9	8.6	53	25	88.8	7.5	71	22	68.8	2.3	11
15	9.0	9.0	76	30	65.6	1.7	8	34	61.1	9.3	16
3	8.4	6.8	40	23	58.8	15.0	65	26	57.9	2.7	31
11	5.9	46.3	25	14	48.9	34.6	41	10	48.0	7.7	35
9	5.5	14.0	54	24	46.1	26.0	136	30	46.7	2.7	24
2	4.6	6.5	51	27	42.7	14.2	39	20	43.1	6.4	21
20	2.7	31.3	102	16	32.6	14.5	53	21	25.6	14.2	82
8	2.1	15.3	56	21	29.4	9.7	34	6	23.4	1.3	7
16	1.3	3.8	28	15	27.0	32.2	222	4	16.3	3.8	34
17	0	5.5	33	12	25.4	26.6	165	13	16.0	17.4	51
19	0	4.0	26	8	15.1	19.5	40	1	12.7	50.7	51
				20	13.9	12.2	17	5	11.6	4.8	28
				11	12.9	47.3	189	12	8.8	29.9	84
				9	5.2	42.0	40	3	8.8	18.4	138
				New Jersey:				16	3.7	9.1	20
				42	88.9	3.0	13	18	3.7	22.7	111
				41	86.1	4.7	20	11	2.9	39.2	122
				50	85.5	10.7	18	New Jersey:			
				47	84.8	26.2	20	50	94.3	14.5	68
				45	84.5	16.2	23	48	92.3	6.5	59
				48	83.1	5.2	30	39	90.0	12.7	53
				51	79.0	23.2	158	49	90.0	42.5	284
				44	77.4	17.5	40	38	85.8	3.9	26
				43	72.7	21.0	93	45	85.5	4.6	30
				38	63.2	3.7	8	47	84.6	21.1	47
				37	60.1	31.0	117	41	82.2	5.8	31
				49	59.6	17.5	69	43	71.0	7.9	65
				36	57.2	27.2	100	51	59.1	31.6	168
								42	58.4	15.4	19
								36	47.8	21.3	118
								37	46.9	34.5	171
								46	32.9	35.0	252

Such comparisons are shown in table 3, where the orchards in Virginia and Maryland have been grouped for the survey in 1937

and those in Virginia, West Virginia, and Maryland have been arranged in one series and those in New Jersey in another series for the surveys in each of the years 1938 and 1939.

From this arrangement it becomes evident that in each series the orchards with the higher rates of parasitization tend to have a lower fruit infestation than the orchards with the lower rates of parasitization. This is true for both values of fruit infestation. However, this relationship lacks uniformity when individual orchards are compared, since there are a number of orchards of low parasitization and low fruit infestation, such as Nos. 17 and 19 in 1937, and an occasional orchard of high parasitization and high fruit infestation, such as No. 49 in 1939.

It becomes desirable, therefore, to examine the data more critically to determine whether there is any significant relation between the parasitization observed and the measures of fruit infestation. For this purpose the orchards in each of the five series in table 3 were divided into two classes, one of high and the other of low parasitization. The point selected for separation was usually the midpoint of the observed range for the series.

If parasitization were not related to fruit infestation, the means of the classes of high parasitization would approximate the means of their paired classes of low parasitization, and the mean of lower fruit infestation would be likely to occur as often in one class as in the other. As shown in table 4, this situation does not exist. In each comparison between classes the mean fruit infestation of the class of high parasitization is lower than that of the corresponding class of low parasitization. This in itself is good evidence that there is an inverse relationship between parasitization and fruit infestation. The standard errors for the class means show not only that the differences between the classes are all in the same direction but also that they are large enough to be significant in 5 of the 10 class comparisons. When the significance of the class difference of the 5 series is calculated, it is indicated that the probability of these differences being significant is slightly more than 100 to 1 for the percentage of fruit infested and slightly less than 100 to 1 for the number of injured fruits per tree. It is concluded, therefore, that in this series of observations parasitization is inversely related to fruit infestation and the higher rates of parasitization produced correspondingly lower rates of fruit infestation.

TABLE 4.—*Averages of fruit injury for classes of high and low parasitization in the series of observations in orchards included in table 3*

Orchard series	Second-brood larvae parasitized	Observations	Total injured fruits	Injured fruits per tree
	Percent	*Number*	*Percent*	*Number*
Virginia and Maryland in 1937	46.9–82.2	6	5.3±1.1	35±9
	0 – 9.0	10	14.3±4.4	49±8
Virginia, West Virginia, and Maryland:				
1938	58.8–98.6	8	4.5±1.7	25±10
	5.2–48.9	11	25.3±3.8	89±22
1939	43.1–84.8	11	3.4± .9	17± 3
	2.9–25.6	11	19.2±4.7	66±13
New Jersey:				
1938	77.4–88.9	8	13.3±3.1	40±17
	57.2–72.7	5	20.1±4.7	77±19
1939	71.0–94.3	9	13.2±4.1	74±27
	32.9–59.1	5	27.6±3.9	146±38

In table 4 the mean percentage of fruit infestation appears to be more closely correlated with parasitization than the mean number of injured fruits per tree. For the percentage value of the fruit injury in the Virginia-Maryland and the Virginia-West Virginia-Maryland series, the mean infestation in the orchards of high parasitization ranged from about one-third to about one-sixth of the mean infestation in those of lower parasitization. In the New Jersey series the mean range was smaller, a condition that may well have been due to the smaller range of observed parasitization in the series surveyed.

SUMMARY

During 1937, 1938, and 1939 a total of 51 orchards, located in 11 peach-growing districts in Virginia, West Virginia, Maryland, and New Jersey, were surveyed to determine whether there was a correlation between parasitization of twig-infesting larvae of the oriental fruit moth (*Grapholitha molesta* (Busck)) and subsequent infestation of the fruit. Comparisons were made (1) by arranging orchards in 5 classes according to percentage of parasitization, (2) by considering individual orchards, (3) by considering individual orchards over successive years, and (4) by grouping orchards by year within a given area.

It was found that, when the comparisons were limited to one season and a restricted district in which the conditions affecting fruit moth abundance, other than the parasitization measured, were reasonably uniform, high parasitization of second-brood twig-infesting larvae was followed by correspondingly low fruit infestation, with evidence that this relation was significant. This condition existed each year and throughout the area surveyed, but was more evident in 1938 and 1939 than in 1937, and in the orchards in Virginia, West Virginia, and Maryland than in those in New Jersey. When the observations were compared in any other manner, factors other than the observed parasitization, and apparently associated with the year and the locality, were sufficiently important to obscure the effect of second-brood parasitization in reducing fruit infestation.

These data support the findings of Daniel and his associates in Niagara County, N. Y., and of Yetter and Allen in Burlington County, N. J., that parasitization of twig-infesting larvae is an important factor in controlling infestation in ripe peaches.

THIAMINE IN CROWN GALL AS MEASURED WITH THE PHYCOMYCES ASSAY [1]

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INTRODUCTION

A better understanding of the growth factors that may exert an inciting influence on pathological cell growth seemed desirable for clarifying this diseased condition. Such factors can be studied with considerable facility by using crown gall, caused by *Phytomonas tumefaciens* (Smith and Town.) Bergey et al. This organism has appeared, in preliminary trials by McIntire, Riker, and Peterson (*24*),[2] to be relatively rich in thiamine, a substance known as a growth factor for many organisms, including higher plants. It seemed important, then, to determine whether there is a quantitative relation of thiamine to crown gall development.

The literature dealing with the role of thiamine in the growth processes of fungi and higher plants, respectively, has been reviewed by Schopfer (*45*) and Lilly (*19*); and by Bonner (*3*). However, certain papers that form the background of the present study are mentioned briefly.

Thiamine was demonstrated as a growth factor for higher plants, independently, by Kögl and Haagen-Smit (*16*), Bonner (*4*), and Robbins and Bartley (*32*). This work was done with excised embryos and roots in vitro. An increase in dry weight of some green plants as an effect of added thiamine has been reported by Bonner and Greene (*7*, *8*), Bonner and Bonner (*2*), and Bonner (*5*). Arnon (*1*) and Hamner (*13*) failed to confirm this beneficial effect on whole plants. Thiamine has been supposed (*29*) to function in respiration as a precursor of part of an enzyme system. No work has been found dealing with the effect of thiamine on pathological growths, such as crown gall. However, the requirement of thiamine by certain fungi makes possible its measurement.

The role of thiamine as a growth factor for *Phycomyces blakesleeanus* Bgf. was demonstrated, independently, by Schopfer (*36*) and Burgeff (*9*). The use of this fungus in a quantitative test for thiamine was proposed by Schopfer (*39*, *40*). Schopfer has done the pioneer work on the development of the *Phycomyces* assay, and the research of other investigators has been based largely upon his findings. It must be borne in mind that *Phycomyces blakesleeanus* is responsive not only to thiamine but also to the pyrimidine and thiazole fractions together (*20*, *34*, *42*, *44*, *47*), and to cocarboxylase (*20*, *48*), the pyrophosphoric acid ester of the vitamin. Hence, in estimating the thiamine content of plant tissues or extracts with the *Phycomyces*

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[2] Italic numbers in parentheses refer to Literature Cited, p. 108.

method, the values obtained may be high because of the action of these other factors (cf. Schopfer, *37*, *41*, *45*, *46*).

Factors other than thiamine which may affect the vegetative growth of *Phycomyces* are of prime importance in assay work since the objective is that the dry weight of the mycelium produced shall be proportional to the available thiamine. The basic assay medium, then, should contain optimum concentrations of everything needed for the growth of *Phycomyces*, except thiamine. Accordingly thiamine, if limiting, might be determined quantitatively by the growth magnitudes of the fungus.

A synthetic medium containing 10 percent glucose, 0.1 percent asparagine, and inorganic salts was devised by Schopfer (*39*). He later (*42*) reduced the glucose content to 5 percent, then (*45*) to 3 percent. Bonner and Erickson (*6*), and McClary (*22*) used 10 percent glucose and 0.4 percent asparagine. Robbins (*28*) employed 5 percent glucose with 0.05 percent asparagine; also media with twice these concentrations. Robbins and Kavanaugh (*34*) used 10 percent glucose with 10 percent asparagine and later (*35*) reduced the asparagine to 1 percent. It is evident that there is lack of uniformity in the concentration of carbon and nitrogen sources used by various workers for the growth of *Phycomyces*. However, the relation of such food sources to growth and the desirability of standardization for assay purposes are recognized.

The interaction of constituents of the medium on the dry-weight yield of *Phycomyces* was studied by Schopfer (*38*). He found a pronounced increase in the dry weight of the mats with an increase in asparagine (0.05 to 4.0 percent), and a different optimum concentration of thiamine for each concentration of asparagine. The glucose content of the medium (1.0 to 15.0 percent) had no effect on the final dry weight of the mycelium when an excess of thiamine and 0.1 percent asparagine were present. Schopfer (*43*) obtained the best growth of *Phycomyces* on a medium of pH 4.0 or 5.0. There was a pronounced decrease in yield at pH 6.0.

The relation of various factors to the growth of *Phycomyces* was studied by Burkholder and McVeigh (*10*). With 4.0 and 8.0 gm. per liter of asparagine, and thiamine at 1×10^{-6}M, glucose was limiting up to quantities of 80.0 or 100.0 gm. per liter. At lower levels of asparagine content, 40.0 gm. per liter of glucose gave maximum growth. Definite increases in growth were obtained as the asparagine content was raised, if 20.0 gm. per liter or more glucose was present. They stated: "It appears that a medium containing 40 gm. per liter of glucose and 4 gm. per liter of asparagine permits good growth, although somewhat higher concentrations yield even more dry matter." Cultures were found to grow best at a pH of 3.5 to 4.6, and a temperature of 15° C.

In none of the reported experiments was material assayed under the varied environmental conditions to determine their effects on the assay results. All workers, of course, have not been primarily concerned with the use of *Phycomyces* as an assay organism. It does not seem necessarily true that conditions which give maximum growth in the presence of an excess of crystalline thiamine will be most favorable for assay use. With the assay, suboptimal concentrations of the vitamin must be used to get an increase in fungus dry weight corresponding

to the increase in thiamine content of the medium. The unknown to be assayed must also be added to the basic medium in quantities which will yield mycelium in this linear range. Thiamine, crystalline or in the unknown material, must always be the limiting factor. In assay work, then, one is much less interested in the conditions inducing the most possible growth in a given volume of medium than in the slope of the growth curve. Thus it seemed necessary to determine the effects of various cultural conditions on the assay results, and then to adapt the assay method to the present problem.

The present studies, therefore, have included (1) adaptation of the thiamine assay to the present problem, (2) comparison of thiamine values of crown gall and healthy tissues, and the influence of various factors on these values, and (3) determination of the thiamine content of cultures of a virulent and an attenuated strain of *Phytomonas tumefaciens*. Such studies gave promise of clarifying the relationship of the plant growth factor thiamine to crown gall development.

An abstract of some of the present work has already been published (*15*).

MATERIALS AND METHODS

The Bonnie Best variety of tomato plants was used for crown gall inoculations, except where stated otherwise. The plants were grown individually in 4- or 6-inch pots in the greenhouse at approximately 24° C. They were inoculated when 6 to 8 inches tall by a needle puncture in each of three central internodes. A highly virulent strain (the A6 culture of Hendrickson, Baldwin, and Riker (*14*) of *Phytomonas tumefaciens* was used unless stated otherwise. Sterile punctures were made in control series of similar plants. Tissue samples for thiamine determinations included (1) galls from all of the three inoculation points, (2) stem segments of inoculated plants from the topmost gall to 1 inch below the terminal bud, (3) analogous segments from check plants, (4) mature, healthy leaves from inoculated plants, and (5) similar leaves from check plants. Any variations in this procedure are stated. Each sample included material from five or more plants. The temperature chambers described by Riker, Henry, and Duggar (*26*) were used in determining the effect of temperature on the thiamine content of inoculated and healthy plants.

The *Phycomyces* growth method was used for the determination of thiamine in the various tissues. Fresh or frozen-dried (*12*) tissue samples were used. The fresh samples were collected between 9 and 10 a. m., cut into about 5-mm. segments, and used immediately. Frozen-dried tissues were thoroughly ground with a mortar and pestle and used when needed. Extracts of the plant materials were employed since attempts to assay quantities of whole tissues gave very inconsistent results between duplicate samples, confirming results obtained by Schopfer (*41*).

Dry-weight determinations were made with duplicates of each sample assayed.

Two methods of extraction, with water and alcohol as the solvents, were used. Materials were water-extracted by autoclaving them for 15 minutes at 15 pounds' pressure in water 100 or more times their dry weight. The extract was then filtered or centrifuged, depending on the density of the original material, and the filtrate or supernatant

liquid used for the assay. In alcohol extraction, a volume of 50-percent ethyl alcohol equivalent to the water in the other method was added to the material. The suspension was agitated continuously for 1 hour with an electric stirrer, filtered, and the material extracted again with a duplicate volume of fresh alcohol. After filtering the second suspension, the 2 filtrates were combined and concentrated under vacuum almost to dryness. The concentrate was brought to desired volume with distilled water. This solution was used for the assay. Both extraction methods were found suitable for the present studies since the calculated thiamine values obtained with water and alcohol extracts of samples of the same material were similar. Water extraction was employed in most of the work because of its simplicity.

The culture of *Phycomyces blakesleeanus* (+strain) used throughout these studies was secured from Prof. Leon H. Leonian. Stock cultures were maintained on the following medium: Thiamine (Merck's Betabion), 10.0 gamma; Bacto-dextrose (Difco), 5.0 gm.; amino acid mixture (*17*) (*d*-arginine, *d*-glutamic acid, *l*-aspartic acid, 2 parts each; *dl*-alanine, glycine, 1 part each), 0.5 gm.; ammonium nitrate, 0.5 gm.; potassium dihydrogen phosphate, 0.5 gm.; magnesium sulphate, 0.5 gm.; calcium carbonate, 1.0 gm.; Bacto-agar (Difco), 20.0 gm.; distilled water, 1,000.0 ml. Seventy-five-milliliter portions of this medium were added to 6-ounce bottles, autoclaved, slanted, and seeded with a spore suspension of *Phycomyces*.

The basic assay medium was prepared double strength, with magnesium sulphate, 0.5 gm.; potassium dihydrogen phosphate, 1.5 gm.; zinc, 0.2 p. p. m.; iron, 0.2 p. p. m.; manganese, 0.02 p. p. m.; Bacto-dextrose and nitrogen as indicated; distilled water to make 500.0 ml. The pH was adjusted to 5.5 with sodium hydroxide. To 50 ml. of this medium was added thiamine solution or plant extract, as the case might be, and the volume was then brought to 100 ml. with distilled water. Twenty-five-milliliter portions of this solution were added to each of four 250-ml. pyrex flasks, giving a quadruplicate series for each concentration. In a few experiments, as noted, duplicate series of 25-ml. portions in 250-ml. flasks were employed. The check series included concentrations of crystalline thiamine from 0 to 0.3 gamma per 25 ml. of medium. Plant extracts to be assayed were always included in two or three concentrations. The flasks were autoclaved at 15 pounds' pressure for 15 minutes. The spores for seeding the flasks were obtained from two stock cultures that had grown for 2 weeks at room temperature. With a hooked transfer needle the sporangia and sporangiophores were removed from the stock cultures and agitated in a tube containing 10 to 15 ml. of sterile distilled water. A drop of this spore suspension was used to seed each flask in a single series. Preliminary experiments indicated that, within a wide range, the amount of inoculum had no effect on the final yield of the fungus. The flasks were kept at 23° C. for selected incubation periods.

At the end of the incubation period, the mycelial mats were removed and thoroughly washed in water. This was done best by pouring distilled water down the side of the flasks so that the mycelium was turned over and over. When the flask was filled the mycelium was removed to an empty flask and again washed. The process was carried through three times, thus washing each mat in about three-quarter liter of

distilled water. The mats were then dried to constant weight at 75° C., and weighed to the nearest milligram. The weight of the mat in each of the four flasks was determined separately, and the average weight per flasks was used in plotting the check curve and in calculating the thiamine content of an unknown. "Check curve" refers to the curve obtained by plotting the concentration of crystalline thiamine against the dry weight of *Phycomyces* produced. Thiamine values for the two or three concentrations of a given unknown were averaged to obtain the final value. Determination of the calculated thiamine value of an extracted unknown was as follows:

$$\frac{\frac{V}{v}B}{W} = \text{gamma of thiamine per gram dry weight of tissue extracted}$$

Where:

V = total volume of extract

v = volume of extract contained in 25 ml. of medium

B = gamma of thiamine per 25 ml. of medium which corresponds to the weight of mat of the unknown, as determined from the check curve

W = gram dry weight of the sample extracted

Example:

Where 2 gm. dry weight of tissue (W) is extracted with 200 ml. of water (V), 2.5 ml. of the extract (v) is included in 25 ml. of medium, and the dry weight of fungus mat produced corresponds to that produced by 0.1 gamma of crystalline thiamine (B) in 25 ml. of medium, then $\frac{\frac{200}{2.5}0.1}{2} = 4$, or gamma of thiamine per gram dry weight of tissue extracted.

ADAPTATION OF ASSAY METHOD

Various factors were studied before the assay was satisfactorily adapted to the present study. These included (1) some variations in the nitrogen and dextrose of the basic medium, (2) the growth period for *Phycomyces*, and (3) the validity of the assay. Alcohol extracts of the same lot of frozen-dried, tomato gall tissue were used unless stated otherwise. The flasks were incubated for 7 to 8 days, or as stated.

EFFECT OF VARIATIONS IN NITROGEN AND DEXTROSE

The source of nitrogen employed by most investigators for the growth of *Phycomyces* has been asparagine. Not a synthesized compound, it may vary between samples and contain impurities which necessitate repeated recrystallizations from water or alcohol. It is more expensive than synthetic glycine. Glycine (*18*, *36*, *43*, *49*) and aspartic acid (*18*) have been shown to be practically as good as asparagine for the growth of *Phycomyces*. Leonian and Lilly (*18*) recommended glycine for assay media. Burkholder and McVeigh (*11*) concluded: "As a source of nitrogen, recrystallized asparagine may be preferable to glycine for thiamin assays with *Phycomyces*." However, no direct comparison of assay results obtained with the use of media containing each of these nitrogen sources was found. If

glycine should prove feasible for assay work, it would thus be preferred over asparagine.

To determine the possibilities of using asparagine, aspartic acid, and glycine, each as the sole nitrogen source in the basic medium, they were employed at various concentrations. The *l*-asparagine (Pfanstiehl, C. P.) was recrystallized from distilled water three times to remove any thiamine present as an impurity. Glycine (Pfanstiehl, C. P.) and *l*-aspartic acid (Eastman) were not treated. The dextrose concentration was varied with asparagine and glycine. Thiamine values of gall extracts assayed on these different media are given in table 1. Only the values in a single experiment are directly comparable since a different extract was used for each. Water extracts were used in experiments 3 and 4.

TABLE 1.—*Some changes in the nitrogen and dextrose of the basic medium in relation to assay results*

Experiment No.	Nitrogen source	Nitrogen concentration per liter [1]	Dextrose concentration per liter	Thiamine per gram of dried tissue
		Gram	*Gram*	γ
1	Asparagine	2.0	25	5.7
			50	5.3
			100	5.1
		4.0	25	6.4
			50	6.0
			100	6.0
2	Aspartic acid	2.0	50	5.8
		6.0	50	5.5
3	Glycine	2.27	25	4.3
			50	4.3
			100	4.9
4	Glycine	1.14	50	5.7
		2.27	50	5.2
		4.54	50	6.6

[1] On a total nitrogen basis, 2.0 gm. of asparagine is approximately equal to 4.0 gm. of aspartic acid, or 2.27 gm. of glycine.

Doubling the asparagine content of the medium gave a slightly higher assay value with each concentration of dextrose, as shown in experiment 1 of table 1. The difference at each dextrose concentration is of about the same magnitude as the widest variation between concentrations of the same extract, and thus is probably on the borderline of significance. Aspartic acid (experiment 2, table 1) gave no consistent increase in assay values with increase in concentration. Glycine (experiment 4, table 1) at 4.54 gm. per liter, however, gave a value 25 percent higher than at a concentration of 2.27 grams per liter. This corresponds with the results from the use of asparagine in experiment 1, though the increase in assay value is slightly greater. The slightly increased values at the higher nitrogen concentrations did not seem to justify a 100-percent increase in the nitrogen supply of the basic medium. The dry weight of the fungus mycelium in flasks with 0.2 to 0.3 gamma thiamine per 25 ml. was increased with the higher concentrations of asparagine and glycine. There was no such increase with aspartic acid. Thiamine was limiting in all these experiments; therefore, pronounced increases would hardly be expected (cf. Schopfer (*38*)).

The data in experiments 1 and 3 of table 1 show that increasing the dextrose concentration in the range employed, with either asparagine

or glycine as the nitrogen source, has no pronounced effect on the assay results of gall tissues. The differences between the values in either experiment are within the range of widest variation between concentrations of the same extract. The lower values with glycine may be due to the different extract employed. Higher concentrations of dextrose increased the dry-weight yield of the fungus with asparagine in the presence of 0.2 and 0.3 gamma thiamine per 25 ml., and the highest concentration of plant extract (equivalent to 0.2 gamma thiamine per 25 ml.). There was no such increase with glycine. A concentration of 50 grams of dextrose per liter seemed in excess but not inhibitory, and so was used in all later assays.

It was thus demonstrated (table 1) that with thiamine limiting, as it must be for assay purposes, increases in the concentration of nitrogen and dextrose within the ranges employed did not affect the assay results.

Glycine, aspartic acid, and asparagine were each demonstrated (table 1) to be suitable nitrogen sources in the basic assay medium. To have an exact comparison of the three, it was necessary to assay samples of a single extract on media containing each as the sole nitrogen source. The data from such an experiment are recorded in table 2. The assay values with glycine and asparagine as nitrogen sources are almost identical. Aspartic acid gave an average value slightly higher than either of the others. Glycine, then, seemed as suitable a nitrogen source for the basic medium as asparagine, or aspartic acid, and was preferred because of its purity and relatively low cost. Therefore, glycine was used in all later assays, at 2.27 grams per liter.

TABLE 2.—*Thiamine values of a plant extract as affected by different nitrogen sources in the basic medium* [1]

Nitrogen source	Nitrogen concentration per liter [2]	Plant extract [3] per 25 ml. of medium	Thiamine in dried tissue	
			Per gram	Average
	Gram	*Milliliter*	γ	γ
Glycine	2.27	1.25	5.4	5.1
		2.5	4.7	
		3.75	5.1	
Aspartic acid	4.00	1.25	5.8	6.4
		2.5	6.9	
		3.75	6.4	
Asparagine	2.00	1.25	4.8	5.3
		2.5	5.4	
		3.75	5.7	

[1] Dextrose at 50 grams per liter was employed with each.
[2] Concentrations are approximately equal on a total nitrogen basis.
[3] Extract of 2.5 grams of gall tissue contained in 250 milliliter of water.

EFFECT OF GROWTH PERIOD

The shortest time that allows maximum growth of *Phycomyces*, at all concentrations of thiamine and plant extract employed, seemed desirable for assay work. Schopfer (*38*, *43*) found that maximum growth occurred in 7 to 9 days in 20 to 25 ml. of medium at 23° C. Some reduction in fungus weight was usually evident after 14 days, with 0.4 to 64.0 gamma of thiamine per liter. Burkholder and McVeigh (*10*) stated: "No loss in weight of the fungus occurred at 25° [C.] even at the end of thirteen days." Their initial concentration of thiamine was 10^{-7} molar.

Two experiments were performed to determine the incubation period most suitable for assay. In the first experiment, duplicate flasks of each concentration of the check thiamine series and of the plant extract series were removed after 5, 6, 7, and 8 days' growth. In the second experiment duplicates were removed at 6-, 7-, 8-, and 9-day intervals. The data of the second experiment, plus the fifth-day data of the first, are recorded in table 3 as the average dry weight of mycelium per flask. The results at 6, 7, and 8 days were similar in the two experiments.

TABLE 3.—*Growth quantities of Phycomyces as related to time period in media with different concentrations of crystalline thiamine and plant extract, alone and combined*

Thiamine in 25 ml. of medium (γ)	Extract [1] in 25 ml. of medium	Average dry weight of fungus per flask at given time (days) after seeding				
		5	6	7	8	9
	Milliliter	*Milligram*	*Milligram*	*Milligram*	*Milligram*	*Milligram*
0.0	0	0	1	0	0	0
.05	0	20	23	26	19	20
.1	0	25	38	42	39	37
.2	0	23	72	77	80	77
.3	0	32	95	110	99	105
.75	0	41	155	160	147	157
1.5	0	45	159	167	161	170
2.25	0	38	161	167	164	172
.0	5	39	54	----------	58	56
.0	10	78	94	101	99	101
1.5	5	117	174	175	177	177
1.5	10	----------	199	190	184	197

[1] 250 milliliter water extract of 1 gram of cold-dried, tomato gall tissue.

The maximum growth of *Phycomyces* in these trials was reached 7 days after inoculation. There was a very rapid increase in growth of the fungus between the fifth and sixth day after seeding. No decrease in fungus weight was evident at 9 days after seeding, except with the lower concentrations (0.05 and 0.1 gamma per 25 ml.) of thiamine. Since assays had been terminated after 8 days' growth in most of these preliminary studies, it seemed desirable to compare the thiamine values obtained at 7 days with those obtained at 8 days.

In several assays, duplicate flasks were removed at 7 and at 8 days and the thiamine values of extracted tissues determined at each of these intervals. The comparative thiamine values (gamma per gram) of five extracts, each determined at 7 and 8 days, respectively, were (1) 5.3 and 4.9, (2) 11.4 and 11.9, (3) 3.1 and 2.9, (4) 8.2 and 7.9, and (5) 12.2 and 12.2. The extracts were of different plant tissues, hence the variation in magnitude of thiamine content. These data show that the difference between assay results determined at 7 and at 8 days is negligible. All later assays were terminated at 7 days because of the convenience of the 1-week interval.

VALIDITY OF ASSAY

The validity of the *Phycomyces* assay is demonstrated to some extent by the data in table 2. Three concentrations of the same extract were used with all of the 3 media. These concentrations contained thiamine within the quantitative range (0.05 to 0.3 gamma per 25 ml.) of the check curve. If thiamine were the only factor

in the extract which limited the growth of *Phycomyces*, the 3 extract concentrations in a single medium should give identical calculated thiamine values. However, the values were not always identical; but neither did they show a consistent increase toward the highest or lowest concentration of the extract. Later experiments showed both more and less variation than was evident in table 2. It seemed unlikely that factors other than thiamine in the extract would influence the growth of *Phycomyces* to exactly the same degree as this vitamin. Hence, the fact that the thiamine values, determined from each of 3 concentrations of the same extract, were similar indicated that the assay was valid for the extracts used. Part of the deviation was probably due to manual error. Further evidence indicating the presence of interfering factors was obtained when combinations of crystalline thiamine and plant extracts were used.

In a medium containing an optimum concentration of crystalline thiamine (i. e., 1.5 gamma per 25 ml., under the conditions of this experiment), plus plant extract, the vegetative growth of *Phycomyces* at 5 days after seeding was pronouncedly increased over that in the same medium containing the thiamine alone (table 3). The fungus mat produced by the combination of thiamine and plant extract exceeded the combined weights produced by the two when employed separately. The higher concentration of plant extract, alone, exhibited a similar but not so pronounced stimulation of early vegetative growth. The cause of this stimulation may have been similar to the "factor Z" reported by Robbins (*30*, *31*) and Robbins and Hamner (*33*). Regardless of the cause, it seemed doubtful if the stimulation of early vegetative growth affected the assay results since the effect was obscured by the seventh day in media containing extracts which gave values within the quantitative thiamine range (table 3). Robbins (*31*) stated: "Whether factor Z affects the dry weight of the mature mycelium of *Phycomyces* when thiamine is present in limited quantity is uncertain." Where thiamine was not limiting, however, the stimulating effect of extracts on maximum growth continued through 9 days (table 3).

The combination of plant extract with an optimum concentration of crystalline thiamine gave a greater yield of mycelium at 6, 7, 8, and 9 days each than did the thiamine alone (extracts plus thiamine, table 3). The increase was greater with the higher concentration of plant extract. This experiment has been repeated with the same results. Since thiamine was not limiting, there must have been another factor in the extract which brought about the increased growth. Perhaps this was the same factor that stimulated early vegetative growth. However, this factor should not influence the assay unless it is evident when combinations of thiamine and plant extract are employed within the quantitative range.

Assuming that the growth of *Phycomyces* on the basic assay medium is determined by the thiamine added, it would be expected that crystalline thiamine and plant extract in combination would produce an effect equivalent to their combined thiamine values when supplied within the range of quantitative sensitivity. This idea was tested in several experiments. The results of 4 of these trials are presented in table 4. In the first trial there was an exact additive effect. Crystalline thiamine and plant extract, in combination, produced a

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growth response identical with that of a crystalline thiamine quantity equivalent to the sum of their thiamine values. In the other 3 trials, however, the actual thiamine values of the combinations were less than expected on the basis of a purely additive effect. In no case was there evidence of the "other factor" discussed in preceding paragraphs. The cause of these lower-than-expected values, though unknown, may be an inhibiting or toxic factor, or factors, in the extract (*11*).

TABLE 4.—*Effect on the growth of Phycomyces of combinations of crystalline thiamine and plant extracts within the range of quantitative response*

Trial No.	Source of extract	Thiamine value [1] of extract in medium (per flask)	Crystalline thiamine in medium (per flask)	Expected values [2]		Actual values [3]	
				Total thiamine in medium (per flask)	Dry weight of fungus (per flask)	Total thiamine in medium (per flask)	Dry weight of fungus (per flask)
		γ	γ	γ	*Milligrams*	γ	*Milligrams*
1	Fresh tomato galls	0.07	0.1	0.17	63	0.17	63
2	do	.112	.1	.212	74	.19	69
3	do	.045	.1	.145	58	.095	42
4	Dry tomato galls	.103	.1	.203	88	.168	73

[1] The concentration of thiamine on the check curve, which corresponds to the same dry weight of fungu as that produced by the extract.

[2] On the assumption that thiamine plus plant extract would give a growth effect equivalent to that produced by a quantity of thiamine equal to their combined values. For example, in trial 1 the expected thiamine value, 0.17 gamma, is the sum of the thiamine value of the extract (see footnote 1), 0.07 gamma, plus the crystalline thiamine added, 0.1 gamma. The expected growth value, 63 mg., is the weight of fungus produced per flask with 0.17 gamma of thiamine per 25 ml., as determined from the check curve.

[3] Actual thiamine value is the thiamine concentration corresponding to the same dry weight of fungus on the check curve.

Such inhibiting factors were occasionally evident with the highest concentrations of the extract series in later experiments. The degree of inhibition varied greatly between experiments. Conditions which determined the presence or absence of these factors are unknown. In 6 of the 65 experiments made during this study, toxic materials in the tomato extracts prevented sufficient growth of *Phycomyces* for the collection of significant data.

The validity of the *Phycomyces* assay was checked against 2 other procedures. Identical results were secured with this and a chemical method based on azo dye formation in assaying water extracts of rye germ.[3] However, of the 2 procedures, the *Phycomyces* assay was much more applicable to measuring low concentrations of thiamine such as are found in rye middlings. Likewise the *Phycomyces* assay and the "chick" assay (*23*) gave comparable results, as discussed later.

ASSAY METHOD AS ADAPTED

As a result of these preliminary experiments, the basic medium for later assays contained 50.0 gm. per liter of Bacto-dextrose, 2.27 gm. per liter of glycine, and the mineral salts as listed earlier. The cultures were incubated for 7 days at 23° C. Water extracts of fresh plant tissues were used, unless stated otherwise. Other phases of the method were unchanged.

[3] IHDE, A. J. STUDIES ON SOME OF THE MINOR COMPONENTS OF THE RYE GERM. 1941. [Ph. D. thesis. Copy on file in the University of Wisconsin library, Madison, Wis.]

As representative of data secured in later assays, results with crystalline thiamine and two plant extracts of the December 9, 1940, assay are given in table 5. The calculated thiamine values of the two

TABLE 5.—*Representative data from the Phycomyces assay of December 9, 1940*

Materials included per 25 ml. of basic medium	Dry weight of fungus per flask					Calculated thiamine values [1]	
	Replicates				Average	From each extract concentration	Average
Crystalline thiamine (γ):	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	γ	γ
0	3	3	1	2	2		
0.05	27	28	26	26	27		
0.1	45	47	45	45	46		
0.2	78	75	79	80	78		
0.3	111	106	113	103	108		
Crown gall. extract (milliliters): [2]							
5	39	44	38	40	40	7.7	7.7
10	64	65	73	67	67	7.6	
Control-stem extract (milliliters): [3]							
5	26	30	29	26	28	2.4	2.7
10	54	56	64	54	57	3.0	

[1] Per gram dry weight of tissue extracted.
[2] A 100-ml. water extract of 2.5 gm. of fresh tomato gall tissue (8.6 percent, dry weight).
[3] A 100-ml. water extract of 5.0 gm. of fresh, control, tomato stem tissue (9.4 percent, dry weight).

concentrations of each extract were almost identical—illustrating again the dependability of the assay. Extract of the control stems showed the most variation, with a difference of 0.6 gamma between the two calculated thiamine values. The widest variations between replicate flasks were less than 10 percent in most cases. These larger variations were probably due to manual error. The data from the crystalline thiamine series in table 5 may be compared with those in figure 1.

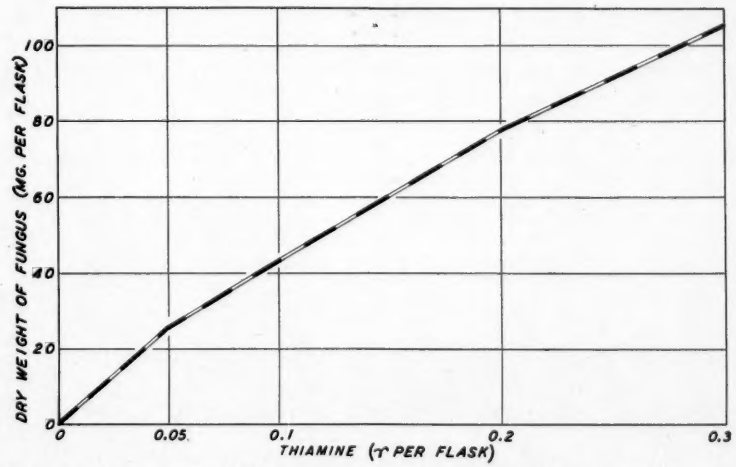

FIGURE 1.—The relation of the growth of *Phycomyces* to the concentration of crystalline thiamine in the glycine medium. Each 250-ml. flask contained 25 ml. of medium.

The relation of crystalline thiamine concentration to the growth of *Phycomyces* is graphically recorded in figure 1. Points on the curve were established from the average data of all (*31*) check curves in which these particular concentrations of thiamine were used. The data were obtained during the period from August 1940 to April 1941. Individual curves varied only slightly from the average. The nearly linear relation of the fungus weight to thiamine concentration in the basic medium is evident from these data. Thus, the check curves met experimental requirements.

EXPERIMENTAL RESULTS

THIAMINE IN CROWN GALL AND HEALTHY TISSUES

The thiamine concentration in gall and stem tissues was determined in numerous experiments with greenhouse tomatoes, three experiments with greenhouse sunflowers, one with field marigolds, and two with field tomatoes. Typical data from greenhouse tomato plants are given in table 5. In this case the concentration of thiamine in gall tissue was nearly three times that in the control stems. Most of the data are omitted because of their similarity to those presented. Preliminary experiments thus showed the thiamine content of crown gall tissue to be consistently higher than that of healthy stem tissue from analogous regions of check plants.

The consistently high thiamine concentration in gall tissue led to further experiments aimed at clarifying the relation of thiamine to gall development. If thiamine were a causal factor in crown gall development, its concentration in the galls might be expected to increase or decrease as they increased in size. A change in thiamine concentration in the galls might also be associated with a concentration change in other parts of the inoculated plants. To determine these relationships, the thiamine content of the leaves, stems, and galls of diseased plants was determined at intervals up to 5 weeks after inoculation. Leaves and stems of healthy plants were assayed for comparison. Six series of tomato plants, each including diseased and healthy individuals, were used. Determinations were made at such intervals that each value in the composite results, given in table 6, included the averaged data from two or three series of plants.

TABLE 6.—*Thiamine in parts of diseased and healthy tomato plants at progressive intervals after inoculation*

Weeks after inoculation	Thiamine per gram dry weight in—				
	Inoculated plants			Check plants	
	Galls	Leaves	Stems	Leaves	Punctured stems
	γ	γ	γ	γ	γ
1	[1] 7.0	7.5	3.7	7.1	4.1
2	[1] 5.1	6.8	4.1	6.0	5.2
3	7.9	4.2	2.2	5.0	2.9
4	8.8	6.5	4.1	7.3	3.1
5	9.3	5.4	4.0	7.7	4.1

[1] That section of the stem containing the inoculation point was used here since the galls were not sufficiently developed to be separated from the stem tissue.

It appears from the data in table 6 that, at least through the first 5 weeks of development, the gall tissue was higher in thiamine content than either leaves or stems. At 1 and 2 weeks after inoculation the leaves of both inoculated and check plants gave higher values than the "gall"; but the fact must be considered that there was no gall, as distinct from stem tissue, during the first 2 weeks and hence the "gall" tissue assayed contained stem material (fig. 2). However, thiamine

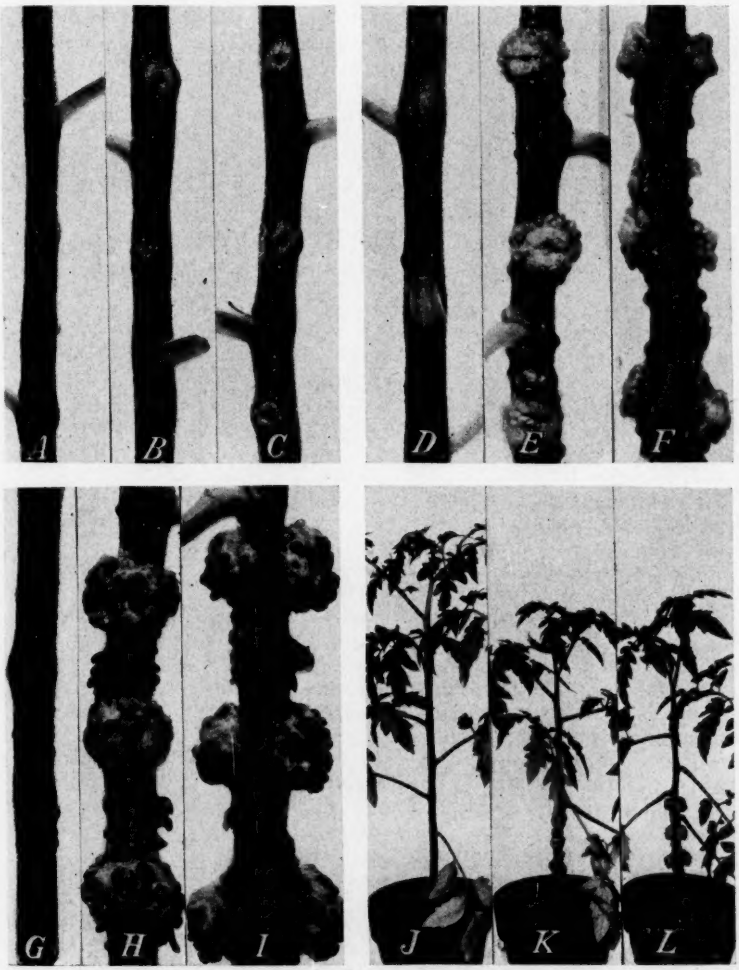

FIGURE 2.—Stages in the development of crown gall on tomato: *A*, *B*, and *C*, 10 days after inoculation; *D*, *E*, and *F*, 20 days after inoculation; *G*, *H*, and *I*, 30 days after inoculation; *J*, *K*, and *L*, whole plants from which the segments *G*, *H*, and *I*, respectively, were taken. *A*, *D*, *G*, and *J*, control plants; all others, inoculated. Inoculated plants (*K* and *L*) are dwarfed in comparison with control plant (*J*). *A*–*I* approximately natural size; *J*–*L* about one-sixth natural size.

was evidently being rapidly accumulated at the inoculation point since the value at 1 week was considerably higher than in stem tissue of either inoculated or control plants. The leaves contained more thiamine than the stems. Values for stem tissues in inoculated and check plants were similar. Values for leaf tissues were similar up to 3 weeks after inoculation. Beyond 3 weeks, however, the leaves of inoculated plants were lower in thiamine concentration than the leaves of control plants of similar ages. The thiamine concentrations in the leaves and stems of control plants and the stems of inoculated plants did not change significantly with an increase in age and size. The galls increased slightly in thiamine concentration from 3 to 5 weeks after inoculation, during the period when true gall tissue was assayed. Though the increase in concentration was slight, the increase in content was much greater because of the increasing size of the galls. There is thus the possibility that the galls were aided in enlarging by the transport of excessive quantities of thiamine from the leaves, hence the decreasing concentration of thiamine in the leaves of inoculated plants as compared with those of control plants.

TOTAL THIAMINE IN INOCULATED AND CONTROL PLANTS

As has been noted in earlier studies, inoculated plants are usually shorter than controls (fig. 2). This stunting becomes more noticeable with increased time after inoculation, correspondingly as the galls increase in size. Thus it seemed that the galls might be depleting other parts of the inoculated plant of necessary factors for growth, e. g., thiamine. The data in table 6, discussed in the preceding section, indicated that on a unit dry-weight basis thiamine was possibly being depleted in the leaves of inoculated plants.

To supplement these data, it seemed necessary to assay whole plants from inoculated and control series and compare their respective thiamine values. The above-ground portions of plants were assayed for thiamine at 4 to 5 weeks after inoculation. From three to five whole plants were used in the preparation of each extract. The thiamine values are thus representative of the entire aerial portions of the plants, not of any specific tissue. The average thiamine value for inoculated plants from three experiments was 5.2 gamma per gram dry weight, and for control plants from identical experiments, 5.7 gamma. Inoculated plants averaged 2.8 gamma dry weight each, making the thiamine content 14.6 gamma per plant. Control plants averaged 3.4 gm. each, giving a thiamine content of 19.4 gamma per plant. The dry weight of both types of plants was roughly 10 percent of the fresh weight. Thus, control plants contained more thiamine though most of the difference was accounted for by the greater weight of the plants.

The similarity of the thiamine values per gram of dry weight for the two types of plants was also shown indirectly as follows: The total weight of the control plants consisted of about 60 percent leaf and 40 percent stem tissues; inoculated plants consisted of about 45 percent leaf, 40 percent stem, and 15 percent gall tissues. Thus the relative amounts of stem tissues in the two types of plants were equal; inoculated stems were shorter but much thicker in the region of the galls. Leaves and galls combined were the same percent of the total weight of inoculated plants as were leaves alone of the control plants.

The gall tissue, then, seemed to have been formed almost entirely at the expense of the leaves. When the thiamine concentrations of the two types of plants was computed from the assay values of the individual parts (galls, leaves, stems) and their respective percentages of the whole plants, given above, the thiamine concentration per gram dry weight of inoculated plants was 5.7 gamma, and of control plants, 5.9 gamma. These theoretical values compare well with the actual ones of 5.2 gamma per gram for inoculated plants, and 5.7 gamma per gram for control plants.

DISTRIBUTION OF THIAMINE IN INOCULATED PLANTS

Concentration of thiamine, as a growth factor, might be expected to be different in meristematic regions of the plant from that in regions that have reached maturity. Crown gall consists largely of tissue of a meristematic nature. Inoculated plants, then, have two aerial meristematic regions: (1) The apical tip, and (2) the region of inoculation. The distribution of thiamine in inoculated plants was studied with particular concern for correlation between the concentrations of this vitamin in the two areas of meristematic activity.

The concentration of thiamine in the following tissues of inoculated plants was determined about 4 weeks after inoculation: (1) Galls, (2) leaves, and (3) sections of the stems above the topmost galls. Stem sections were divided into 1.5-inch segments. The segments from the top (including the terminal bud), central, and bottom areas of the sections were assayed separately, giving three thiamine values for stem tissues. The thiamine concentration per gram dry weight of tissues from four replicates of the above experiment were averaged for the following values: Galls, 8.1 gamma; leaves, 6.8 gamma; top stem segments, 9.6 gamma; central stem segments, 5.8 gamma; and bottom stem segments, 5.6 gamma. The thiamine concentration in the stems thus showed a definite gradient which fell rapidly below the meristematic region.[4] Galls were higher in thiamine concentration than leaves, and both were higher than the mature stem areas but considerably lower than the stem tips. However, the thiamine concentration of the galls did approach that of the stem tips. This similarity may be due in part to the meristematic nature of gall tissue.

THIAMINE IN PLANTS GROWN ABOVE AND BELOW THE MAXIMUM TEMPERATURE FOR GALL FORMATION

It has been shown by Riker (*25*) that crown galls develop well on tomato at 28° C. but fail to develop on this host at 32°. If thiamine were the limiting factor for gall formation, it might be expected that plants grown at 32° would show a deficiency in the vitamin to account for the failure of gall development. A series of plants was inoculated and one-half of them transferred to each of two chambers, regulated to 28° and 32° C., respectively. After 5 weeks the plants were removed and stems, leaves, and galls assayed for their thiamine content. Galls had developed only at the lower temperature. The thiamine values per gram of dry weight for the 28° galls, leaves, and stems, respectively, were 8.4, 7.0, and 4.6 gamma; for the 32° leaves and stems,

[4] After completion of the present manuscript, the following publication appeared reporting a similar gradient in tomato stems: BONNER, J. TRANSPORT OF THIAMIN IN THE TOMATO PLANT. Amer. Jour. Bot. 29: 136–142. 1942.

respectively, 6.9 and 5.1 gamma. A similarly treated series of plants inoculated with a partly attenuated bacterial culture gave corresponding results. Since the higher temperature evidently had no effect on the concentrations in the host plants, thiamine deficiency could not have prevented gall development.

THIAMINE IN GALLS FROM VIRULENT AND PARTLY ATTENUATED BACTERIAL CULTURES

A partly attenuated culture, the A6–6 strain of Hendrickson, Baldwin, and Riker (*14*), of *Phytomonas tumefaciens* erratically produces small galls, a few millimeters in diameter, on tomato. If thiamine were limiting the growth of the A6–6 galls, it might be expected that the concentration of this vitamin would be considerably less in them than in the galls produced by the virulent culture (A6).

A series of plants was inoculated with each of the cultures. Uninoculated, but punctured, plants served as controls. Thiamine assays were made on the various plant parts 8 weeks after inoculation. The thiamine concentration per gram of dry weight of the A6–6 galls was 7.8 gamma, and of the A6 galls, 8.8 gamma. On the basis of thiamine content per gall, the values for A6 galls were much higher because of their relatively greater size. The thiamine concentrations in stems and leaves, respectively, were similar in the two types of inoculated plants, and in control plants.

When tomato stems are inoculated with the A6 strain and, a few millimeters below, with the A6–6 strain, the latter produces galls similar in size to the A6 galls above them (*21*). This effect may be caused by some growth factor which diffuses from the region of the A6 inoculation and accelerates the A6–6 gall development. Galls from two such series of plants were assayed for thiamine 6 weeks after inoculation. The average thiamine value per gram dry weight of the A6 galls was 8.5 gamma; of the A6–6 galls, 8.8 gamma. The thiamine concentration in these A6–6 galls, equal in size to the A6 galls, was only 1 gamma per gram greater than in the A6–6 galls which were alone on the plant and showed practically no development. Thus, the thiamine concentration in galls from the partly attenuated culture was increased but little when they were stimulated to greater growth.

To determine whether thiamine had any of the stimulating properties of the virulent bacterial culture when applied above inoculations of the A6–6 strain, thiamine in lanolin was applied to the decapitated tips of 45 plants inoculated with the attenuated bacteria. One-third of the series was treated with plain lanolin, one-third with 0.3 percent thiamine in lanolin, and one-third with 3.0 percent thiamine in lanolin. After 1 week, the plants were decapitated for the second time and fresh lanolin mixtures applied. The experiment was terminated 4 weeks after the first treatment. There were no observed differences between the thiamine-treated and the nontreated plants in their reaction to A6–6 inoculations. Though stems of the plants, minus the lanolin-covered tips, were assayed for thiamine at weekly intervals, the results were so erratic that it could not be certain how much of the added vitamin reached the inoculation points. Thus, from this evidence, thiamine alone did not seem to possess the stimulating properties of the virulent culture.

COMPARISON OF THIAMINE CONTENT OF VIRULENT AND PARTLY ATTENUATED BACTERIA

The virulent strain (A6) of *Phytomonas tumefaciens* had been shown (*24*) to be relatively rich in thiamine. It seemed desirable, therefore, to compare this strain and the partly attenuated one (A6–6) in regard to their thiamine concentrations. If the A6–6 strain synthesized less thiamine in culture, it might act likewise when introduced into a host plant and so influence the host reaction.

Several cultures of the two strains were prepared for thiamine assay.[5] The bacteria were grown in a synthetic medium (*23*). Two hundred-milliliter portions of the medium were added to 1-liter serum jars and autoclaved. The jars were seeded with the desired bacterial culture and incubated for 5 to 6 days at 28° C. The cultures were aerated with the aid of an automatic shaker for the entire incubation period. At the end of this period the cultures were centrifuged and the cells assayed for thiamine.

The average thiamine value per gram dry weight of cells from seven cultures of A6 was 17.5 gamma, and from five cultures of A6–6, 22.1 gamma. The value for A6 cells is higher than that (12.0 gamma per gram) determined by the "chick assay" and reported by McIntire, Riker, and Peterson (*24*). More rapid growth in the present improved medium and the pyrimidine and thiazole fractions of thiamine, possibly present in the cells, may account for the higher value obtained with *Phycomyces*.

Attempts to assay the cellfree medium failed because of the presence of a substance, or substances, inhibitory to the growth of *Phycomyces*.

DISCUSSION

The lack of uniformity in the conditions under which the *Phycomyces* assay had been used by various workers necessitated some studies on the method preceding its adaptation to the present problem. Though most workers have used asparagine as the source of nitrogen in the assay medium, it has the disadvantage of not being practically available in synthetic form. It must therefore be treated to remove the thiamine which may be present as an impurity. The amount of thiamine and other impurities may also vary between different lots of asparagine. It was in an attempt to overcome these disadvantages that glycine was tried as the nitrogen source. Glycine had previously been reported as suitable for the growth of *Phycomyces* (*18*, *36*, *43*, *49*) and suggested for use in the assay medium (*18*). The present studies demonstrated the applicability of this compound to assay use, and it was preferred to asparagine (1) because of the more nearly linear relation of the weight of the fungus mat produced to the thiamine concentration in the medium (i. e., a more nearly straight "check curve"), (2) because of its relative purity, and (3) because of its relatively low cost. A basic medium containing 2.27 gm. per liter of glycine, 50.0 gm. per liter of Bacto-dextrose, and inorganic salts proved quite satisfactory. The medium is less concentrated than that used by many workers. However, with thiamine limiting, increasing the nitrogen supply gave only slightly higher assay values, and increasing the dextrose supply had no apparent effect on the

[5] Dr. F. C. McIntire gave valuable assistance in the bacterial culture work.

values. Therefore, the more concentrated media, which provide maximum growth with an excess of thiamine, need not be the best for assay use where thiamine must always be limiting.

A growth period of 7 days for *Phycomyces* was the nearest optimum with all concentrations of thiamine (0.05 to 0.3 gamma per 25 ml.) and plant extracts under the environmental conditions used. There was a very rapid increase in growth between 5 and 6 days after seeding. After 9 days' growth there was no decrease in fungus weight except in cultures with very limited supplies (0.05 and 0.1 gamma per 25 ml.) of thiamine. Schopfer (*38*, *43*), and Burkholder and McVeigh (*10*) reported no loss in fungus weight for even longer growth periods when an excess of thiamine was present.

The *Phycomyces* assay seemed to be valid since (1) different concentrations, or quantities, of an extract gave similar calculated thiamine values; (2) crystalline thiamine and plant extract combined exerted a growth influence equal to their combined thiamine values, except where the inhibitive action of the extract seemed evident; (3) "other factors" in the plant extracts which stimulate growth of *Phycomyces*, even with an optimum concentration of thiamine present, were not evident at 7 days in media containing the extracts within the quantitative thiamine range; and (4) thiamine values of bacterial cells compared well with those obtained with the "chick assay" and reported by McIntire, Riker, and Peterson (*24*).

For determinations of thiamine in plant tissues, the *Phycomyces* assay is quite suitable since minute quantities may be detected in relatively crude tissue extracts. The method as used was sensitive to differences of 0.01 gamma of crystalline thiamine per 25 ml. The sensitivity to changes in the plant extracts was less, but still sufficiently quantitative for comparative purposes. Factors inhibiting the growth of *Phycomyces*, as sometimes encountered with tomato extracts, caused the most serious difficulty.

The present studies have shown that thiamine concentration in crown gall tissues was consistently higher than in mature leaf or stem tissues of the same plant or similar tissues from uninoculated plants. Leaves contained more thiamine per unit of dry weight than mature stems, but usually less than galls. Thiamine was present at the inoculation points in almost maximum concentration within 1 week after the bacteria (*Phytomonas tumefaciens*) were introduced. Hence there was not much increase in thiamine concentration during the period of rapid increase in gall size.

As the galls increased in size, thiamine concentration in the leaves of inoculated plants tended to decrease. This, in addition to the much lower percentage of leaf tissue in inoculated plants than in control plants, suggested that the galls were depleting the leaves of a normal complement of thiamine, thus contributing to the dwarfing of inoculated plants.

Two reasons are offered for the relatively high thiamine concentration in the gall tissues. First, the bacterial cells are rich in the vitamin, as shown by McIntire, Riker, and Peterson (*24*) and confirmed in the present studies. It is noteworthy that the number of bacteria reaches the maximum during the first week (*27*) when the thiamine concentration is almost at the maximum. Second, the gall tissue is meristematic in nature and, as was shown in the case of stem tips,

this type of tissue is considerably higher in thiamine concentration than mature or nearly mature tissues.

Thus, the higher thiamine concentration in crown galls than in mature, normal tissues from adjacent regions of the same plant or similar regions of healthy plants, is probably due, in the early stages of gall development, more to the presence of crown gall bacteria than to the meristematic nature of gall tissue. With progressive development, however, the relative importance of these causes is possibly reversed.

Other lines of evidence, discussed under their individual headings, failed to indicate that thiamine played a causal role in crown gall initiation or development. These included comparisons of the thiamine concentration in inoculated and control plants, the effect of temperatures above and below the maximum for gall formation on the thiamine concentration in the host plant, and comparisons of the thiamine concentrations in virulent and partially attenuated bacterial cells and galls produced by these cells.

It appears, therefore, that thiamine alone does not have a causal role in crown gall initiation or development beyond that of any necessary food or growth factor transported to, or produced in, that area of meristematic activity.

SUMMARY

Some relations of thiamine to crown gall development have been studied. The *Phycomyces* assay was used for all thiamine determinations.

Glycine, aspartic acid, and asparagine were compared as nitrogen sources in the basic medium for *Phycomyces*. Glycine was selected as the most suitable source because of its relative purity, its relatively low cost, and the more nearly linear relation of thiamine concentration in the new glycine medium to the dry weight of *Phycomyces*.

A basic assay medium containing 2.27 gm. per liter of glycine and 50.0 gm. per liter of dextrose was most satisfactory.

At 23° C. an incubation period of 7 days gave optimum growth at all concentrations of crystalline thiamine and plant extract employed.

Thiamine concentration in crown gall tissue was consistently higher than in mature, or nearly mature, stem tissues from inoculated or check plants. This relationship was found with greenhouse and field tomatoes, greenhouse sunflowers, and field marigolds as host plants.

Thiamine accumulated in almost maximum concentration at inoculation points within 1 week after treatment, and before macroscopic galls were evident.

The thiamine concentration in galls remained fairly constant from 3 to 5 weeks after inoculation, during the period of rapid increase in gall size, and approached that in the growing tip of the host plant.

Mature leaves contained more thiamine per unit of dry weight than mature stems, but usually less than galls.

The content of thiamine per unit dry weight of the whole aerial portions of inoculated tomato plants was similar to that of comparable portions of control plants. Control plants contained more thiamine, however, because they were larger.

Temperatures above (32° C.) and below (28° C.) the maximum for gall formation on tomato had no apparent effect on the thiamine concentration in various tissues of inoculated plants.

The minute galls produced by a partly attenuated culture of *Phytomonas tumefaciens* contained as high a concentration of thiamine per gram of dry weight as did the large galls produced by the virulent culture.

Stimulation to large gall production, where the attenuated culture was introduced into a stem below inoculations of the virulent culture, had slight if any effect on the thiamine concentration of the resulting galls.

Crystalline thiamine in lanolin failed to produce similar stimulation.

The bacterial cells of the partly attenuated culture, grown on a synthetic medium, contained more thiamine (22.1 gamma) per gram of dry weight than the cells of the virulent culture (17.5 gamma) grown on the same medium.

It appears, therefore, that thiamine alone does not have a causal role in crown gall initiation or development beyond that of any necessary food or growth factor transported to, or produced in, that area of meristematic activity.

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HARDWOOD INVASION IN PINE FORESTS OF THE PIEDMONT PLATEAU [1]

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INTRODUCTION

Forest stands of pure pine are important factors in the wood-using economy of the Carolina and Virginia Piedmont Plateau. Thousands of portable and semiportable mills representing everything from small, part-time, family-operated businesses to fairly large, full-time mills are, for the most part, dependent upon pine stumpage for their existence. Both the farm-woodland owner and the industrial landholder find that ready markets exist for a variety of pine forest products and that it takes hardwoods many more years to yield similar products. The demand not only for lumber but for pulpwood, poles, and a number of minor products has tended to call for a large proportion of pine in the total stumpage utilized locally. Although good grades of hardwood logs are usually accepted at the larger permanent mills, the market for these species, particularly oaks and hickories, has not been as steady as the market for pine. The large furniture industry of the piedmont region finds in nearby forests only a limited number of usable native hardwoods and is dependent on distant sources for sugar maple, birch, cherry, and other standard furniture woods.

Comparison of stumpage values and present growth rates of pines and hardwoods provides further evidence of the importance of pine species in piedmont forest enterprises. Records for 1940 show an average stumpage value of $5.83 per thousand board feet for 466 transactions involving a total of 204.2 million feet of yellow pine, principally shortleaf and loblolly, in the Carolinas and Virginia. From the upland hardwood type, dominated by oaks and hickories, a total of 4.2 million feet of oak stumpage was sold in 52 transactions at an average price of $5.73 per thousand board feet, while 258 thousand board feet of hickory in 9 transactions brought an average stumpage price of $4.16. Hardwood species that dominate the bottom-land hardwood type, on the other hand, have stumpage values equal to that of pine or even higher. Among these species are yellow poplar (*Liriodendron tulipifera*), sweetgum (*Liquidambar styraciflua*), red maple (*Acer rubrum*), black tupelo (*Nyssa sylvatica*), and sycamore (*Platanus occidentalis*), though markets for the last three are still quite limited.

Studies [2] of growth rates in existing stands indicate that pine types are much more productive than the upland hardwood type with which they are commonly intermingled. In the North Carolina piedmont, uncut second-growth sawlog-size stands occupy 25 percent

[1] Received for publication August 14, 1942.

[2] CRUIKSHANK, J. W. FOREST RESOURCES OF THE PIEDMONT REGION OF NORTH CAROLINA. U. S. Forest Serv. Appalachian Forest Expt. Sta. Forest Survey Release 6, 55 pp., illus. 1940. [Processed.]

of the forest land. For the various forest types within this condition class, current annual growth per acre in board feet was found to be 300 for shortleaf pine, 331 for loblolly pine, 199 for bottom-land hardwoods, and 159 for upland hardwoods. Partly cut second-growth stands occupy about 18 percent of the forest lands; within this class, current growth was 189 board feet for shortleaf pine, 215 for loblolly pine, 202 for bottom-land hardwoods, and 124 for upland hardwoods.

Present evidence therefore indicates that the pine types not only grow at a much faster rate than the upland hardwood types, but also produce wood of greater value. Attainment of maximum returns from upland piedmont forests consequently appears to require maintenance of as high proportions of pine as are economically and silviculturally feasible. This is not necessarily true for the stream margins and other moist locations characteristic of the piedmont bottom-land hardwood type. Here growth is somewhat slower than in the pine types, but the lower production is offset by the greater values of some of the dominant hardwood species.

In the Carolinas and Virginia, the piedmont region, with a total land area of about 26 million acres, lies between the foothills of the Appalachian Mountains on the west and the level Coastal Plain to the east. A rolling, upland country, sloping gently eastward from elevations of 1,000 to 1,200 feet in the Appalachian foothills to 400 or 500 feet at its eastern margin, this region is favorable to agriculture because of its topography, a 6-months' growing season, and a 40- to 50-inch annual rainfall.

Long-continued and widespread farming has had a marked influence in modifying forest types and conditions. Slightly more than half of the land surface of this region is occupied by forest, of which more than 70 percent is classified as pine types. The cycle of land clearing, cultivation, soil erosion and impoverishment, and finally abandonment, has created conditions particularly favorable to the development of pure pine stands. Exposure of mineral soil, as in abandoned fields, is a requirement for the best germination and early survival of pine. The dispersal of the light, wind-borne seed from pines growing in adjacent forest completes the conditions under which the pines could gain ascendency over their common plant associates. Since three-fourths of the present forest area of the piedmont region was in cultivation at some time in the past, there is little reason to wonder at the high proportion of pine types now present.

During the past generation both land clearing and abandonment of land for farm-crop production have abated. Agriculture has become relatively stabilized on the better lands. Woods burning has been reduced by the direct activities of State and Federal forest-conservation agencies, and by the educational programs of these and other public and private organizations. Under such conditions natural trends in the development of forest associations might be expected again to become operative. For this reason the presence of numerous hardwoods in the understory of pure pine stands and their conspicuousness in the growth following logging have led many to suppose that nature tends toward stands of pure hardwoods or of mixed hardwoods and pine, rather than pure pine.

If quantitative evidence can establish that the understory hardwoods now present in pure pine types are thriving, then the assumed

hardwood invasion can be more fully substantiated as an active trend already operative in the territory—a development which may seriously reduce the proportion of pine in future forests and have a marked effect upon silvicultural practices. Late in the summer of 1938 a study was made to collect quantitative data that would establish the widespread presence or absence of this trend and provide specific measurements of its character.

OBSERVATIONS BY OTHER WORKERS

Early literature dealing with the natural resources of the piedmont region provides strong evidence that the original forests were dominated by hardwood species. In 1858, Wilkes (*13*)[3] made especial reference to the culled-over oak forests of the piedmont and predicted the disappearance of hardwood timber there. Bruce (*3*) and Curtis (*4*) mentioned the predominance of oaks and hickories, the former stating that one-fourth of the original forests of Virginia were walnut. However, because hickory at that time was classed as a kind of walnut, it is assumed that a large proportion of this abundant "walnut" was *Carya* species. Hale (*5*) and Parkins (*8*) mentioned pine species as subordinate to hardwoods in old-growth stands. In 1897, Pinchot and Ashe (*9*) referred to the remaining original forest stands on piedmont uplands as being composed of oaks and hickories with an admixture of shortleaf pine in some places. Second-growth forests, however, had "pine for the forest body generally, and hardwoods as subordinate."

More recently, general observations by ecologists have led to the hypothesis that the extensive second-growth pine forests of the Piedmont are beginning to revert to hardwoods. Weaver and Clements (*10*), and Wells (*11*, *12*) have stated this point. Quantitative data substantiating the existence of such a trend have been published by Billings (*2*) who made detailed studies of seven old-field shortleaf pine stands occurring in Durham County. Even in the sandy soils of the longleaf pine type, Heyward (*6*) found strong evidence of replacement of pine by hardwoods where the forests had not been burned.

METHODS OF STUDY

The study was limited to stands of the two pines of the piedmont region most important commercially—shortleaf pine (*Pinus echinata*) and loblolly pine (*P. taeda*). A random selection of 40 piedmont counties in the Carolinas and Virginia was first made and sample stands within each of them were studied. It was necessary to exercise some choice of study areas in order to obtain samples of a wide range of stand ages, sites, and densities for both species. Sampling was further limited to areas where pine made up at least 90 percent of the overstory stem count and where neither grazing nor light cutting had taken place within 5 years, and no extensive cutting or thinning within 10 years. Bottom lands and swampy areas were excluded because they are not typical of the region and occupy a relatively small portion of the forest area. Within these limitations the selection of sample stands was objective, to prevent any partiality toward pine stands either with or without hardwood understories.

[3] Italic numbers in parentheses refer to Literature Cited, p. 127.

In forest stands meeting the requirements named, detailed data were recorded on 117 plots (fig. 1). Of these, 65 were in the shortleaf type and 52 in the loblolly type. Most of the plots were one-fourth acre in size, but a few situated in dense, young stands were only one-tenth acre.

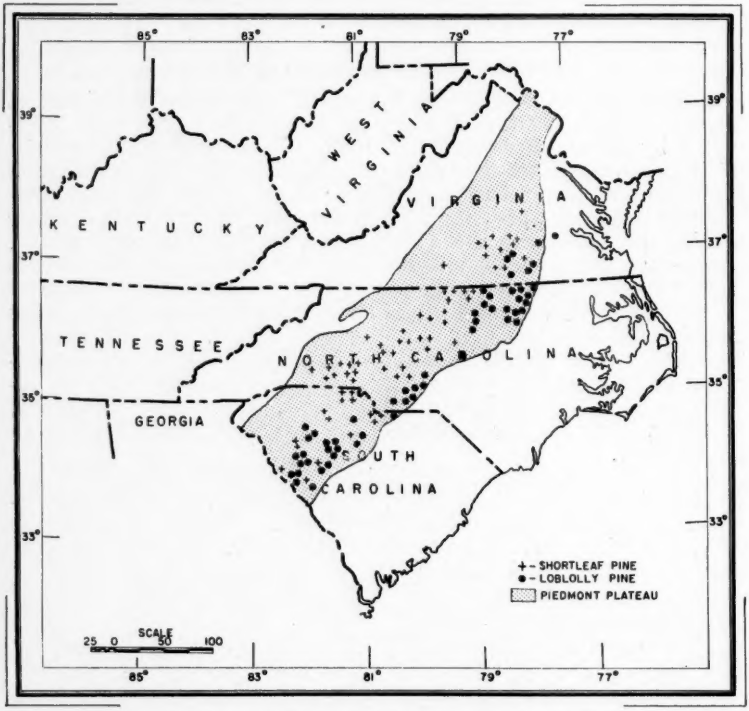

FIGURE 1.—Location of sample plots and extent of Piedmont Plateau in the Carolinas and Virginia.

On each sample plot a stem count of the woody vegetation, including both shrubby and arborescent forms, provided data for the three measures of the understory used, namely: (1) The percent of all hardwood stems represented by the climax species, oaks and hickories, (2) the number of oaks and hickories per acre, and (3) the number of stems of pine reproduction per acre. From measurements of sample trees, the pine overstory of each plot was described by age, site index, and density index. Methods of determining density of stocking in loblolly pine were those developed by MacKinney and Chaiken.[4]

[4] MACKINNEY, A. L., and CHAIKEN, L. E. VOLUME, YIELD, AND GROWTH OF LOBLOLLY PINE IN THE MID-ATLANTIC COASTAL REGION. U. S. Forest Serv. Appalachian Forest Expt. Sta. Tech. Note 33, 30 pp., illus. 1939. [Processed.]

Density index is defined by these authors as the ratio (expressed as percent) of the observed number of trees of all species per acre to the number expected in fully stocked stands of loblolly pine. In the coastal region fully stocked loblolly stands of the following average diameters at breast height are assumed to contain trees per acre, respectively: 5.0 inches, 924; 10.0 inches, 283; and 15.0 inches, 142. Site index is defined as the average height in feet attained by dominant and codominant trees in such fully stocked stands at the age of 50 years.

For shortleaf pine, density of stocking was based on similar but as yet unpublished density criteria. The number of years having elapsed since the latest fire was carefully estimated from such evidence as the age of sprouts arising from fire-killed hardwood trees and the number of annual rings in the callus growth over fire scars found in cut stems.

The plots were then grouped into two categories: First (termed "unburned"), those which had not been burned over for 10 years or more; and second, those which had been burned at least once within 10 years. Data from each group were tested in a series of multiple regressions for relationships between measures describing the understory of each plot and measures describing the pine overstory. The multiple regression method was used because it provides a ready means for detecting the direction of trends and the testing of their significance. Graphic comparison of average regression lines with plotted residuals indicated that for most of the tests relationships were linear or nearly so. Because significant average trends rather than accurate predicting devices were sought, no attempts were made at refinements that would recognize the presence of curvilinear relationships in the few cases where this apparently occurred.

RESULTS FOR UNBURNED STANDS

The majority of the plots had not been burned within the preceding 10 years, and are referred to as unburned. It was from these unburned stands that the most significant and clear-cut results were obtained. Results from the burned stands were much less conclusive, probably because of the comparatively limited amount of data and the impossibility of reconstructing accurate fire histories.

Of the three criteria used to describe the pine stand—age, site index, and overstory density—the first is likely to be the most revealing in any study of long-time changes in the hardwood understory. This is clearly shown in table 1. The importance of the other two criteria lies in their conceivable effect on hardwood understories and hence on the correlation existing between age of the pine stand and the understory. Both density and site, however, as well as age, have significant effects upon understory pine reproduction. Such are the important generalities drawn from the study. They will be developed more fully in the following discussions.

Because oaks and hickories are commonly accepted as climax species of the region, their representation (percent of total stems) in the hardwood understories of pine stands is of particular significance. Furthermore, any consistent differences in their representation under pine overstories of varying age is strongly indicative of natural trends. Figure 2 shows that in unburned stands of both shortleaf and loblolly pine, oaks and hickories are abundant in hardwood understories, and also that their proportionate number increases with the age of the pine overstory. The climax species make up about 17 percent of the hardwood understory in 20-year-old shortleaf pine stands. This proportion rises steadily to approximately 42 percent in 90-year-old shortleaf. In loblolly pine stands the trend is the same but at a lower level. Strong evidence is thus provided that oaks and hickories are able to increase in competition with associated understory hardwoods.

TABLE 1.—*Summary: Correlation of dependent variables describing understory with independent variables describing pine overstory*

Dependent variable describing understory	Species and stand condition	Significant change with increase in overstory in respect to—		
		Age	Density index	Site index
Representation of oak and hickory stems in total hardwood understory.	Shortleaf:			
	Burned	None	None	None.
	Unburned	Significant increase.	do	Do.
	Loblolly:			
	Burned	do	do	Do.
	Unburned	Highly significant increase.	do	Do.
Number of understory oak and hickory stems per acre.	Shortleaf:			
	Burned	None	do	Do.
	Unburned	Highly significant increase.	do	Do.
	Loblolly:			
	Burned	do	do	Do.
	Unburned	do	do	Do.
Number of stems of pine reproduction per acre.	Shortleaf:			
	Burned	do	do	Highly significant increase.
	Unburned	Highly significant decrease.	Significant decrease.	Significant increase.
	Loblolly:			
	Burned	None	None	None.
	Unburned	do	Significant decrease.	Significant increase.

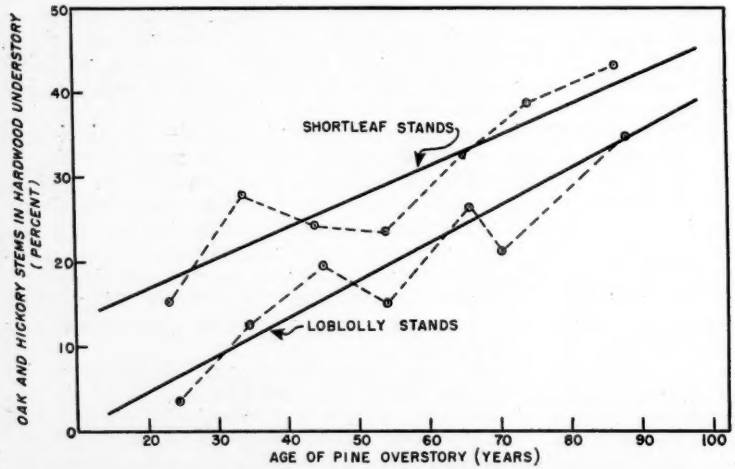

FIGURE 2.—Correlation between age of pine overstory and representation of oaks and hickories in the hardwood understory for stands unburned for at least 10 years. Plotted points are residuals.

Figure 3 shows that increasing representation of oaks and hickories is accompanied by an absolute increase in their numbers, in both shortleaf and loblolly pine stands. For the former the average number of understory oaks and hickories was about 200 per acre at 30 years of age, increasing to approximately 1,000 stems per acre at 90 years. For loblolly pine the average number of understory oaks and hickories was approximately 100 and 600 stems per acre at 30 and 90 years,

respectively. These statistics showing absolute and relative increases in numbers of oaks and hickories substantiate the general observation that invasion by these climax hardwoods is now under way in the pine forests of the piedmont region.

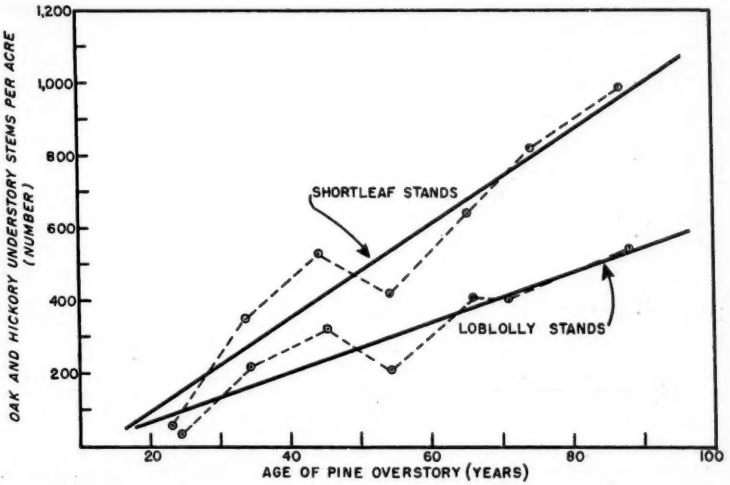

FIGURE 3.—Correlation between age of pine overstory and number of understory oak and hickory stems per acre for stands unburned for at least 10 years. Plotted points are residuals.

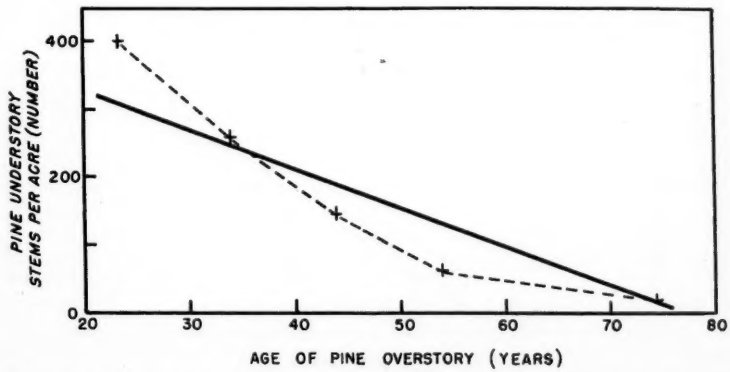

FIGURE 4.—Correlation between amount of understory pine reproduction per acre and age of pine overstory for shortleaf pine stands unburned for at least 10 years. Plotted points are residuals.

Relationships between pine reproduction and the pine-overstory characteristics already mentioned provide another measure of the importance of hardwood invasion. The pine reproduction showed a trend opposite to that of understory hardwoods. For shortleaf pine,

figure 4 shows that the number of understory pine stems per acre decreases as the parent stand grows older and that average stands beyond 70 years of age contain very little pine reproduction. Figure 3 has shown that at this age 700 to 800 oaks and hickories per acre will be

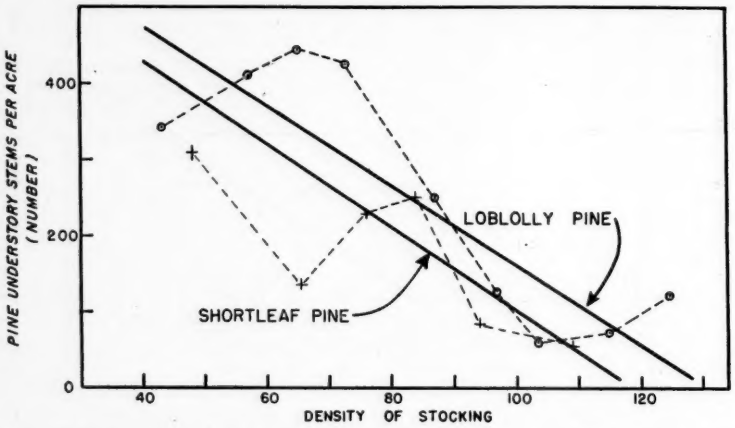

FIGURE 5.—Correlation between overstory density of pine stands and amount of understory pine reproduction per acre in stands unburned for at least 10 years. Plotted points are residuals.

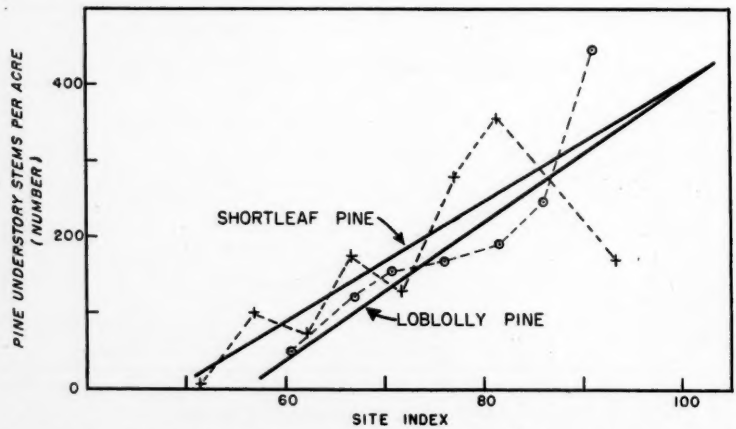

FIGURE 6.—Correlation between site index of pine overwood and amount of understory pine reproduction per acre for stands unburned for at least 10 years. Plotted points are residuals.

present. Understory reproduction of loblolly pine differed from that of shortleaf pine in its relation to age of the parent stand, since there was a slight increase in loblolly seedling count as the main-stand age advanced. From a statistical viewpoint, however, the amount of

loblolly pine reproduction in unburned stands is not significantly correlated with overstory age (table 1). In other words, the average number of loblolly pine seedlings per acre does not differ greatly with respect to various ages of the overstory. For the stands studied, the average number per acre was approximately 200.

Aside from their differing relations with overstory age, the amounts of understory pine reproduction in both shortleaf and loblolly pine stands react similarly to variations in site index and overstory density. The reproduction of both species decreases sharply in numbers per acre as density of the overstory increases (fig. 5). This gives further evidence of the intolerance to shade of these species and their general disinclination to grow in uneven-aged stands except those which are very open. Figure 6 shows that this trend may, however, be modified by site, the amount of reproduction of each species increasing with the site index.

Discussion of the development of understories in relatively unburned pine stands has thus far been confined to average trends. More specific comparisons of understory conditions for various combinations of overwood age, site, and density are presented in figure 7.

For shortleaf pine, figure 7 indicates that in stands 40 to 50 years of age and older, the climax hardwoods will outnumber pine reproduction except under the lower overwood densities. It is also apparent that the excess of climax hardwoods over pine reproduction is likely to be greater on the poorer shortleaf sites than on the better ones. The Forest Survey has shown that for shortleaf pine stands in North and South Carolina, site index 60 is the one of most frequent occurrence. It follows that the advance reproduction in stands of sawlog size, say those older than 45 years, is quite likely to be dominated by oaks and hickories rather than by the reproduction of the parent stand.

For loblolly pine the trends are somewhat different. The number of understory stems of this species is more nearly equal to the number of oaks and hickories. Nevertheless, on sites 60 and 70 oaks and hickories outnumber pine reproduction in the better-stocked older age classes. According to Forest Survey records, site index 70 is of most frequent occurrence in loblolly pine stands of the Carolinas. Consequently, advance reproduction dominated by climax hardwoods may be commonly expected in many of the better-stocked loblolly stands, although figure 7 indicates that the preponderance of oaks and hickories over pine in the understory will not be so great in loblolly as in shortleaf pine stands. On loblolly pine sites 80 and 90, particularly the latter, the amount of pine reproduction exceeds that of climax hardwoods except in the oldest and densest stands.

In the foregoing discussion comparisons have been made between the amounts of pine reproduction and climax hardwoods present in the understories or advance reproduction of pine stands. The climax hardwoods, oaks and hickories, were used in these comparisons because it seems reasonable to expect that pine reproduction will suffer more severe and persistant competition from them than from secondary hardwoods.

Although for certain combinations of overwood conditions, as already shown, oaks and hickories do not exceed pine stems in the understory, when the number of secondary hardwoods is added (fig. 2) the total is much greater than that for both pine species for practically

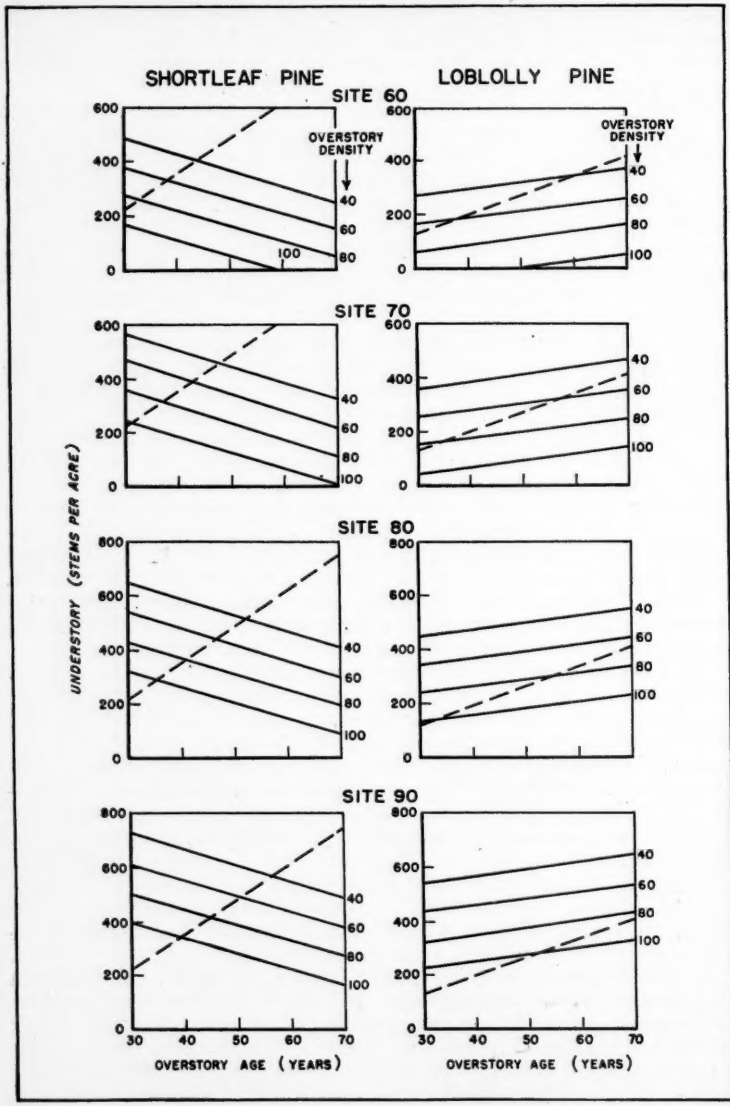

FIGURE 7.—Comparison of understory pine with understory oak and hickory stems in pine stands for various combinations of overwood age, density, and site. Broken lines show average numbers of oak and hickory stems, solid lines average numbers of pine understory stems. Stands unburned for at least 10 years.

all overstory combinations. Since the competition of secondary species may fully equal that of the oaks and hickories in limiting pine establishment and growth during the first few years after cutting, the combined effect of primary and secondary hardwoods is of first importance.

RESULTS FOR BURNED STANDS

It is interesting to compare the foregoing evidence of successional trends in the relative absence of fire with findings in stands burned at least once during the past 10 years. Because sample-plot records from such burned stands were available only in limited quantity, however, and because both fire histories and intensity of individual fires probably varied greatly, few significant relationships were discovered.

As shown in table 1, no evidence of hardwood succession in burned stands of shortleaf pine was found. Neither the number of stems nor the percent of climax species in the understory was correlated with overstory age, density, or site. Apparently the fires in these shortleaf stands had the effect of halting the trend toward hardwoods. Climax species in the understory were not eliminated, however, but were present on an average in about half the numbers found in unburned stands. Pine reproduction in the burned stands, it may be added, was more abundant on the better sites, as it was also in the unburned stands.

The burned-over loblolly pine stands studied gave evidence of successional trends similar to those in unburned stands. Both the proportion of climax species in the understory and their absolute numbers were found to increase significantly as the pine overwood grew older. Moreover, in these respects there was almost no difference between burned and unburned stands. Figures 8 and 9 when compared with figures 2 and 3 illustrate this result. Evidently the fires that occurred in the loblolly stands had very little effect on the understory hardwoods.

Effects of fire on understory pine reproduction also differed between stands of the two pine species. In shortleaf pine stands, the number of understory pine stems was found to increase as the overwood grew older. This relationship, shown in figure 10, is in direct contrast to the trends of understory pine reproduction in unburned stands, as may be seen by referring to figure 4.

In burned loblolly pine stands all normal relationships between amount of pine reproduction and overstory characteristics appeared to be upset by the fires. Pine reproduction had not been eliminated in these burned stands, but was present in from one-half to two-thirds the amounts found in similar unburned stands.

To summarize the results from the burned stands, it can merely be stated that fire was apparently favorable to maintenance of shortleaf pine, but showed opposite effects in loblolly stands. No evidence gathered in the study was helpful in explaining this inconsistency. It is believed that the shortleaf and loblolly pine stands examined had generally dissimilar fire histories, and that the differences in fire effects are due primarily to such variations. It did not appear possible to reconstruct the fire histories accurately by the ex post facto procedures necessarily used.

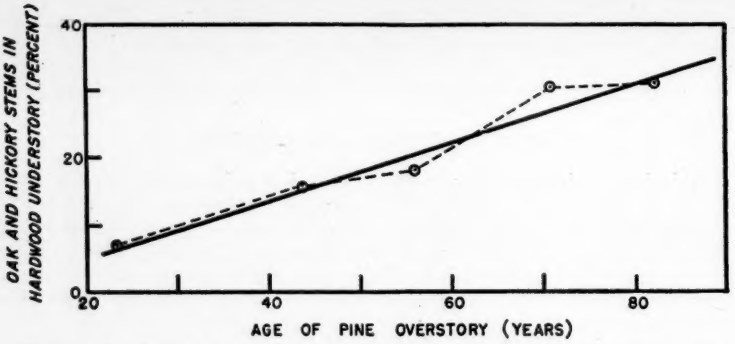

FIGURE 8.—Correlation between age of pine overwood and representation of oaks and hickories in the understory for loblolly pine stands burned at least once during the past decade. Plotted points are residuals.

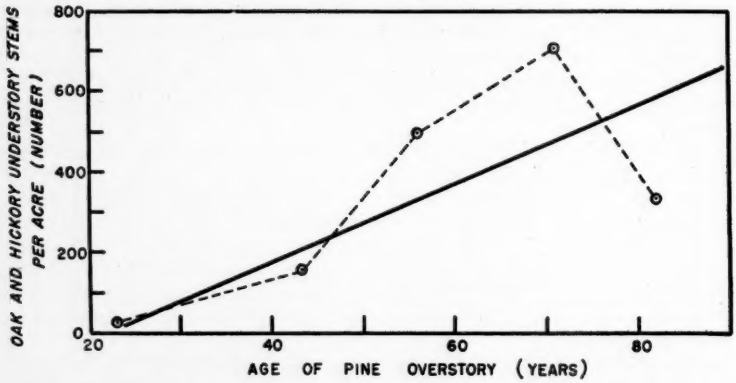

FIGURE 9.—Correlation between age of pine overstory and number of understory oak and hickory stems per acre for loblolly pine stands burned at least once during the past decade. Plotted points are residuals.

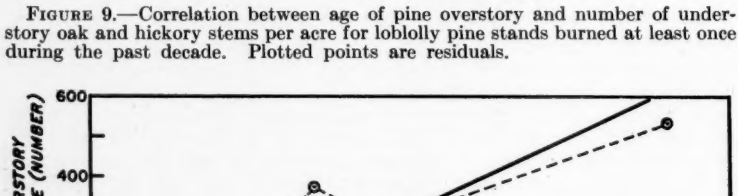

FIGURE 10.—Correlation between age of pine overstory and amount of understory pine reproduction per acre for shortleaf pine stands burned at least once during the past decade. Plotted points are residuals.

IMPLICATIONS OF RESULTS IN FOREST MANAGEMENT

The establishment of hardwood invasion as an aggressive successional trend in shortleaf pine stands and a less aggressive but nevertheless distinct trend in loblolly pine carries far-reaching implications with respect to the management of these stands. Not only do the oaks and hickories in the understory outnumber the pines on many unburned areas, but these climax species are only a part of the total number of understory hardwoods. Many of the associated hardwoods play a subordinate role in mature hardwood stands, but they may be equally effective competitors of pine in juvenile stages. Not only do these hardwoods compete with smaller numbers of growing pine seedlings for light, moisture, and nutrients, but their presence creates a condition unfavorable to the ready establishment of pine reproduction. The mantle of hardwood leaf litter tends to prevent contact of the pine seed with the mineral soil, which is essential for good germination and early survival of the pines. On the other hand, a litter of hardwood leaves forms the best natural seedbed for good germination and early survival of heavy-seeded species such as the oaks (*1*, *7*).

Although it is entirely probable that it will be advantageous to allow replacement of pine to take place on some areas, it is at present impossible to determine which these are, except for the more obvious locations in well-drained stream bottoms and other moist sites where the more valuable hardwoods are usually present in some degree. Investigation of soil characteristics, which have been markedly changed by several generations of cultivation and subsequent depletion, probably offers the best avenue for solution of this problem.

It is also probable that no practicable forest-management measures can entirely control a natural trend so pronounced and persistent as the one described. The drastic measures that originally accomplished control of hardwoods and creation of pure pine stands, namely, extensive land clearing and cultivation, cannot be considered practical in timber production. It is possible, however, that workable methods for partial control of hardwoods and maintenance of high proportions of pine can be developed.

One possibility that suggests itself is the use of fire. Controlled burning, for a variety of purposes, has long been a controversial subject in the South. Although this study provides some indication that fire may favor establishment of pine, the evidence is not at present sufficiently clear-cut and consistent to justify recommendations of burning as a general practice.

For the immediate future, the most promising measures for maintenance of a high proportion of pine in mixed stands are found in the methods of taking the final harvest and in the silviculture applied to subsequent new growth.

Where forestry is practiced, two general methods are customarily considered for harvesting mature pine saw timber, i. e., extremely heavy or clear cuttings by the strip, spot, or seed-tree method, and lighter or selective cuttings. Following seed-tree cuttings, no trees remain except the few chosen, and all or part of these may be removed after reproduction is established. In clear cutting, it is expected that renewal of the stand will be accomplished by seed already on the ground or dispersed from uncut bodies of timber purposely left adjacent

to the cutting area. The objective of the clear-cutting method is to produce even-aged stands so distributed that annual or periodic harvests of financially mature portions of the forest represent a sustained yield from a given property. The method envisions thinnings, improvement cuttings, and salvage cuttings prior to the final cut, for the dual purpose of obtaining intermediate harvests and maintaining the most desirable growth rate of those stand components chosen for the final crop. The basic concept of this method, therefore, is the management of a forest property by even-aged groups and it is usually thought to apply best to species that do not reproduce well in the shade of older trees. The term "clear cutting" is understood to connote the orderly harvest of mature stands with area regulation to keep the rate of cutting within the limits of sustained yield, and carefully planned provision for reproduction.

In the lighter or selective cuttings, individual mature or poor-risk trees are removed throughout an entire cutting area and a comparatively heavy stand remains. The method usually combines essential features of both harvest and improvement cuttings in a single operation, which is repeated at short intervals. Here, renewal of the stand is accomplished by seedling establishment in the small openings created by the selective cutting. The usual concept among foresters is that the use of this method implies the maintenance of an all-aged or many-aged forest, wherein several or many age classes may be present on an area as small as a quarter or even a tenth of an acre. The method is commonly considered as being the best for optimum long-time production of shade-tolerant species. As used here, therefore, the term "selective cutting" applies only to those operations by which mature saw timber is harvested and provision is made for regeneration under a silvicultural policy aimed at development and maintenance of all-aged stands. It does not include thinnings and improvement cuttings made in young stands for intermediate harvests of pulpwood, poles, or piling.

Will these two methods provide equally well for a high proportion of pine in future forests, where aggressive hardwood invasion exists? In the all-aged stands produced by selective cutting it is probable that the loblolly and shortleaf pine overstory would average about 40 in density index and about 70 years in age. The most common site index in shortleaf pine stands is 60. For site index 60, density index 40, and age 70, figure 7 shows that the average understory will be composed of about 250 pine seedlings and 740 oak and hickory stems per acre. Figure 2 shows that oak and hickory stems will make up about 35 percent of all understory hardwoods; accordingly the total number of hardwood stems will be more than 2,100. For loblolly pine of the most common site, which is 70, there will be about 460 pine seedlings and 410 oaks and hickories per acre in the average understory. Figure 2 shows that these climax species will be about 27 percent of all understory hardwoods, totaling 1,500 stems per acre. With hardwoods so far outnumbering pine in the understory, the chances that pine seedlings will occupy each opening made by selective cutting appear poor indeed.

If overstories are maintained at densities higher than index 40, the average amount of understory pine reproduction will be reduced, with the possibility of higher odds against the filling of the openings by the

growth of pine seedlings. If pine overstories are maintained at densities below index 40, the amount of understory pine reproduction increases with probable consequent improvement in the chance that some pine seedlings will grow through the hardwoods into dominant positions. But as overstory densities are reduced the cuttings depart further from the usual concept of selective cutting and approach the more drastic treatments that result in production of even-aged stands.

For the common pine sites of the piedmont region, therefore, this study indicates that selective cutting in pine saw-timber stands may promote the gradual replacement of pine with hardwoods. Because pine reproduction is better represented in understories of stands with low overwood densities and because it is generally conceded that both shortleaf and loblolly pine seedlings develop more rapidly in full light than in shade, it becomes necessary to consider the possibility that some form of drastic treatment, such as clear cutting by the strip, group, or seed-tree system, is more likely to maintain high proportions of pine in future forests than is selective cutting. Clear cutting brings about certain physical conditions that may reasonably be expected to favor maintenance of pine. The complete felling of the heavy pine overstory breaks down a portion of the hardwood understory present. For the removal of the comparatively high volume of logs, more swamping, skidding, and hauling are necessary, still further reducing the hardwoods. These operations also break up the mantle of forest litter and expose mineral soil, creating conditions favoring the germination of pine seed. Complete removal of overhead shade and the reduction of root competition will accelerate the growth of pine after establishment. Under the conditions created by such operations, the new growth of pine starts off on a more equal basis with the hardwoods than when selective cutting is practiced.

When a seed-crop failure occurs in the autumns preceding or immediately following the harvest cut, so that there is insufficient pine reproduction, the hardwoods may get a start of 2 or 3 years. After the establishment of seedlings from a subsequent cone crop, a cleaning several years later to release pine crop trees from hardwood competition may be essential.

Two kinds of stands encountered in shortleaf and loblolly pine types are possibly not in danger of hardwood invasion. One of these is loblolly pine on an unusually high-quality site, and the other occurs in areas where apparently an unusual series of repeated light fires over a long period of years has so reduced hardwoods that their invasion will be very slow. It has already been shown that on loblolly pine sites of 90 or more, the understory will contain a fair percentage of pine seedlings, which will outnumber climax hardwoods except in fully stocked stands of the older age classes. General observation indicates that here pine is frequently able to penetrate through and keep above the hardwood understory. Under these conditions it is possible that either even-aged or all-aged management may maintain the pine in high proportions.

In the second type of stand, where hardwoods have been largely eliminated by light fires, it is again probable that either type of management will maintain high proportions of pine for some time, particularly on the better sites. Under such conditions there will be few, if any, hardwoods to compete with pine seedlings.

Except for the two conditions discussed above, the evidence presented here points to a likelihood that clear-cutting measures will prove superior to selective cutting, where maintenance of high proportions of pine is an objective of management.

SUMMARY AND CONCLUSIONS

Conclusions based on results in stands unburned for at least 10 years may be summarized as follows:

(1) In both loblolly and shortleaf pine stands, the proportion of climax species, oaks and hickories, in hardwood understories increases with age of the pine overwood.

(2) The number of understory climax hardwoods per acre also increases with age of the main pine stand.

(3) Neither the density nor the site index of the pine stand has a significant influence upon the two trends mentioned above.

(4) In shortleaf pine stands, the amount of pine reproduction per acre decreases significantly as the overstory grows older, but under loblolly pine it tends to remain approximately constant for all ages of the overstory.

(5) In both loblolly and shortleaf pine stands the amount of understory pine reproduction per acre decreases with increasing density of overwood, but increases with improvement in the site index.

(6) Differences in age, density, and site of both loblolly and shortleaf pine overstories are associated in several different ways with the amounts of pine reproduction present in the understories. Under certain overstory stand conditions the oaks and hickories will outnumber pine reproduction, and under other conditions the reverse will be true, as shown in figure 7.

(7) The number of combinations of overstory age and density under which climax hardwoods will exceed the amount of pine reproduction is greater on poor than on good sites for both species of pine. In shortleaf the preponderance of climax hardwoods over pine reproduction is greater than in loblolly stands. Hence the trend toward replacement of pine by oaks and hickories appears to be the more aggressive in shortleaf pine stands.

(8) For site index 60 (fig. 7), which most frequently characterizes shortleaf pine stands of the Carolinas, oaks and hickories will outnumber pine reproduction with respect to the majority of possible combinations of overwood age and density. For site index 70 (fig. 7), of most frequent occurrence in loblolly pine stands, oaks and hickories will outnumber pine reproduction only in stands of higher densities.

(9) Climax oaks and hickories represent only a part of the entire hardwood understory of both shortleaf and loblolly pine stands. Figures 2 and 7 indicate that in most of these stands pine stems in the understory will be greatly outnumbered by hardwoods, counting not only the climax species but also the secondary hardwoods, which may be effective competitors of pine during the reproducing years following cutting.

In stands burned at least once during the past 10 years the findings were as follows:

(1) In the burned shortleaf pine stands studied, understory climax hardwoods were present in about half the amounts found in unburned stands. No tendency toward an increase in the representation or numbers of oaks and hickories was found.

(2) Understory pine reproduction in burned shortleaf pine stands showed a marked increase with advancing age of the overstory, a reversal of the trend found in unburned stands.

(3) Climax hardwoods in the understories of loblolly pine stands showed the same trends as those in unburned stands. Fires in loblolly stands apparently had little effect upon the numbers and representation of understory oaks and hickories.

(4) Pine reproduction, although not eliminated, was reduced in burned loblolly stands, and the pine understory had no consistent relationships with overstory density and site.

(5) The study failed to disclose any reasons for the inconsistencies in results for burned stands. Lack of consistency is attributed primarily to wide but undetermined variations in fire histories of the stands studied.

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